

Evaluation of the fungicide treatment with copper oxide and potassium phosphonate solutions for the sustainable management of *P. pinaster* trees infected with *B. xylophilus*

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Abstract

Fungicides induce changes in the plants promising to increase tolerance of *Pinus pinaster* against the pathogenic pinewood nematode (PWN). To test this hypothesis, *P. pinaster* plants were inoculated with the PWN, treated with copper oxide (CO) or potassium phosphonate (PP), and evaluated post-inoculation for: i) the extent of foliar symptoms; ii) nematode density inside stem tissues; iii) proxies for oxidative damage and antioxidant activity, iv) mineral concentration; and v) bacterial diversity. The mortality of infected plants reached 12.5% regardless of the treatment, but plants treated with fungicides, particularly with PP, had significantly lower PWN density (up to 0.61-fold). Plants treated with PP had substantially higher concentrations of anthocyanins at 14 dai than those treated with CO and non-Treated plants (by 1.47-fold), possibly contributing to the lower PWN colonization and degree of foliar symptoms observed. CO and PP led to increased lipid peroxidation at 28 dai (by 1.84- and 1.77-fold), and PP showed higher flavonoids concentration than CO (by 1.37- and 0.49-fold), corroborating its higher potential in increasing plant antioxidative response during infection.

Fungicides also induced significant changes in micronutrient accumulation in plant tissues, resulting in a decrease in Zn and P concentrations in plants treated with either fungicide as compared to infected non-treated plants. Finally, CO treatment increased the diversity of the bacterial communities, while PP decreased microbial biodiversity. Altogether, results suggest that treatment with CO and PP increases tolerance against *B. xylophilus* by promoting the plant antioxidant system, changing the accumulation of essential minerals, and modulating plant-associated bacterial diversity.

Introduction

Pinus spp. is the most widely used genus in industrial forest plantations worldwide (Mbabazi 2011). Maritime pine (*P. pinaster*) is particularly relevant to the European timber industry. Several Western European countries, such as Portugal, Spain, France, and some North African countries (Chupin et al. 2015), produce maritime pine. Despite its economic and social importance, in recent years, its production primarily decreased due to significant losses of forest area and wood volume due to fire and also to the spread of the pinewood nematode (PWN) (Abad et al. 2016), which causes the pine wilt disease (PWD), an exotic pathology of invasive and especially virulent behaviour that causes the rapid death of infested trees. Given the severity of this disease, which has been and continues to be devastating in many countries, and the lack of effective control measures, developing new phytosanitary prevention and control strategies is of utmost urgency. Currently, the control of the PWD relies on the use of pheromone traps against the insect vector *Monochamus galloprovincialis* (Álvarez et al. 2016, Firmino et al. 2017) and breeding for resistance, which have high costs, are time-consuming, and can only be applied to new plantations (Kurinobu 2008, Nose and Shiraishi 2008, Carrasquinho et al. 2018, Menéndez-Gutiérrez et al. 2018). Currently, research efforts against the PWD involve the use of biological control agents, such as ectomycorrhizal fungi that improve plant defences (Nakashima et al. 2016, Chu et al. 2019), or the use of elicitors, such as methyl-jasmonate (MeJA) and salicylic acid (SA), which activate systemic acquired resistance (SAR) or induced systemic resistance (ISR), resulting in higher tolerance to the pathogen (Bari

and Jones 2009, Salas-Marina et al. 2011, Kolosova and Bohlmann 2012, Nakashima et al. 2016, Klessig et al. 2018, Chu et al. 2019, Tripathi et al. 2019, Park et al. 2020).

Using fungicides can also activate the resistance of plants against pests and diseases (Daniel and Guest 2005, Prasad et al. 2017), but their effectiveness against the PWN has yet to be discovered. Copper oxide (CO) is a well-known fungicide used in organic farming against mildew (Cabús et al. 2017), which has shown high antibacterial activity against several parasitic microorganisms, including nematodes (Soli et al. 2010, Burke et al. 2016; La Torre et al. 2018). Copper is a vital mineral and critical component of plant defensive pathways, resulting from its antimicrobial properties and function as a co-factor of several essential enzymes (Borgatta et al. 2018, Elmer et al. 2018, Strayer-Scherer et al. 2018, Mir et al. 2021). This compound has shown *in vitro* nematicidal activity against *B. xylophilus* and other plant pathogenic nematodes (Tan et al. 2013, Mohamed et al. 2019). Likewise, potassium phosphonate (PP), a phosphonic acid salt, is a systemic fungicide that activates SAR and local acquired response (LAR). It promotes the activity of the enzyme phenylalanine-ammonium lyase (PAL), a key regulator of secondary plant metabolites, such as phenols, lignin, phytoalexins, suberin, and compounds derived from cinnamic acid (Astaneh et al. 2018, Kahromi and Khara 2021). Thus, this fungicide could help control the PWD, as the accumulation of metabolites such as soluble phenolics and lignin correlate with higher resilience against the PWD (Moreira et al. 2009, Nunes da Silva et al. 2015, Zas et al. 2015).

When the nematode enters the plant host, it moves and reproduces within the resin canals, causing general oxidative damage that results in visible leaf necroses (Kuroda 2008, Yamada 2008). To counteract the detrimental effects of these oxidative molecules, plants activate several antioxidant enzymes, such as superoxide dismutase, peroxidases, and catalase. Therefore, the elicitation of plant defences before infection through the application of fungicides like CO and PP could improve the tolerance of *P. pinaster* against oxidative damage caused by PWN, owing to their ability to promote plant antioxidant system. In addition, micro and macronutrients also play an essential role in plant tolerance against biotic and abiotic stresses (particularly in resistance to pests and diseases) (Kirkby and Römheld 2004, Bala et al. 2018, Mukherjee et al. 2019, Chan et al. 2021). Therefore, changes in plant mineral composition resulting from fungicide application could affect their response to these stresses (Hossain et al. 2013, dos Santos Silva et al. 2020, Motta-Romero et al. 2021, Parent and Quinche 2021). Although this phenomenon occurs in other species, the impact of CO and PP on pine plants' mineral accumulation and its potential repercussion on plant susceptibility to the PWN is unknown.

Concurrently, plant-associated bacterial communities also play an essential role in the absorption of certain minerals, plant growth promotion, and defence against pathogens (Doornbos et al. 2012, Burketova et al. 2015, Zhang et al. 2018, Gu et al. 2020, Wang et al. 2020). Although the bacterial communities associated with *P. pinaster* and PWN contribute to the development of PWD (Proença et al. 2010, 2017, Roriz et al. 2011, Vicente et al. 2011), the effect of fungicides like CO and PP on the modulation of bacterial diversity has not been evaluated yet.

Thus, this study aimed to assess the effectiveness of CO and PP as tools to induce the tolerance of pine plants against the PWN through the evaluation of i) nematode progression in plant tissues; ii) foliar symptoms and photosynthetic pigments; iii) proxies for plant defensive capability and oxidative damage (anthocyanins, carotenoids, total polyphenolics, flavonoids, and lipid peroxidation), iv) plant-associated bacterial populations, and v) plant mineral profile (B, Cu, Fe, Zn, K, and P).

Materials And Methods

Plant material and experimental design

One hundred and seventeen two-year-old *P. pinaster* plants were grown in the greenhouses of Misión Biológica de Galicia-CSIC (MBG-CSIC, Pontevedra, Spain; 42.4054° N, 8.6426° W). Seeds from French-Landes provenance region were planted in 2L containers filled with peat and perlite (3:1, v:v). The average height and diameter of the plants used were 124 ± 14 and 0.89 ± 0.05 cm, respectively. These plants were transferred to Centro de Biotecnologia e Química Fina-Universidade Católica Portuguesa (CBQF-UCP, Porto, Portugal; 41.1539° N, -8.6733° W), where the experiments took place. These were carried out from April 9th to May 14th 2019 under natural environmental conditions.

Treatments

Seven days before the infection with *B. xylophilus*, the two selected fungicides were applied to a group of 66 pine plants; 33 plants were sprayed with a suspension of 0.2% copper oxide (CO) (commercial product, Nordox copper 75WG Copper oxide 75% ecological) and 33 were treated with a suspension of 0.4% potassium phosphonate (PP) (commercial product, Alexin 75 LS) (both concentrations recommended by the manufacturer). Finally, a group of 33 plants were used as non-infected control plants and 18 plants served as non-treated control plants.

Nematode culture and plant inoculation

Seven days after of fungicide treatment, a virulent strain of *B. xylophilus* (strain 17AS) was used for plant inoculation. The nematodes were maintained in mycoboxes with *Botrytis cinerea* (Pers) mycelia growing in barley seeds at 25 °C for 14 days. Nematodes were extracted from the culturing medium using the Baermann funnel technique (Baermann 1917) for 24 h at 25 °C, and their concentration adjusted so that a solution with 2 000 nematodes in 750 µL of sterilized water was obtained. Inoculation was performed as described by Futai (2003). Briefly, at approximately 20 cm from the top of each plant, leaves were removed from a 3 cm portion of the stem, and transversal cuts were made using a sterile blade. A piece of absorbent paper was placed around the wound, the nematode suspension was pipetted, and parafilm was used to seal the inoculation site. Thirty-three plants of each treatment, i.e., non-treated, treated with CO, and treated with PP, were inoculated with 2.000 PWNs, while an additional group of 18 non-treated plants was inoculated with deionized water (mock-inoculation), resulting in 4 treatments: mock-inoculated non-treated control plants (niCTR), inoculated non-treated controls (iCTR), inoculated CO-treated plants (iCO) and inoculated PP-treated plants (iPP).

Scoring of foliar symptoms and sampling

Eight plants from each treatment were used to evaluate the progression of the disease through visual analysis of leaf foliar symptoms at five different time-points: 7, 14, 21, 28 and 35 days after inoculation (dai). These consisted of wilting and defoliation and were visually assessed on a 0–4 scale: 0 = 0–10% symptomatic leaf tissue; 1 = 11–33%; 2 = 34–66%; 3 ≥ 67%; and 4 = total leaf wilting or defoliation (Sánchez et al. 2005).

Plant sampling was performed at the same time-points (7, 14, 21, 28 and 35 dai). The leaves of 5 plants randomly selected from each treatment were separated from the stems, ground to a fine powder with liquid nitrogen, and used for chlorophyll, lipid peroxidation, total soluble phenols and total flavonoids content and minerals quantification, whereas stems were used for whole-stem nematode quantification and the microbiological analysis.

Nematode quantification

The leaves of plants used for nematode quantification ($n = 5$) were removed, and stems were cut into small portions (*ca.* 0.5 cm). Nematodes were extracted from stems using the Baermann funnel technique for 24 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

For total chlorophyll and carotenoids quantification, the Sims and Gamon (2002) method was used. In brief, 0.1 g of leaf tissue was mixed with 10 mL of cold acetone/Tris buffer solution at 1 M (80:20, v:v, pH = 7.8) and incubated at 4 °C for 24–72 hours, after which samples were centrifuged at 13.000 rpm for 5 minutes. Using the NanoPhotometer™ UV/VIS spectrometer (Implen GmbH, Germany) absorbances were recorded at 470, 537, 647 and 663 nm, and the concentration of pigments was calculated as follows, taking into consideration the sample fresh weight:

$$\text{Anthocyanin} = 0.08173A_{537} - 0.00697A_{647} - 0.002228A_{663}$$

$$\text{Chl}_a = 0.01373A_{663} - 0.000897A_{537} - 0.003046A_{647}$$

$$\text{Chl}_b = 0.02405A_{647} - 0.004305A_{537} - 0.005507A_{663}$$

$$\text{Carotenoids} = (A_{470} - (17.1 \times (\text{Chl}_a + \text{Chl}_b) - 9.479 \times \text{Anthocyanin}) / 119.2$$

For the quantification of soluble phenols and flavonoids, 50 mg of lyophilized leaf tissue was extracted with 1.5 mL of 80% aqueous methanol (v:v) in an ultrasound bath for 20 minutes. The extract was recovered after centrifugation at 15.000 g for 15 minutes.

Total soluble phenolics were determined according to the Folin-Denis' method (Marinova et al. 2005). To 100 µL of methanolic extract, 4.5 mL of ultrapure water and 500 µL of Folin-Denis' reagent were added.

The mixture was vigorously mixed and the reaction allowed to occur for 5 minutes, after which 5 mL of sodium carbonate at 7% (w:v) was added. After incubation at room temperature in the dark for 1 hour, 2 mL of ultrapure water was added to each sample. The absorbances were recorded at 750 nm using a NanoPhotometer™ UV/VIS spectrometer (Implen GmbH, Germany) and the concentration of total soluble phenolics determined using a gallic acid calibration curve.

For flavonoids determination, the aluminium chloride method (Zhishen et al. 1999) was used. To 100 µL of methanolic extract were added 2 mL of ultrapure water and 150 µL of NaNO₂ at 5%. The mixture was incubated for 5 minutes at room temperature. Afterwards, 150 µL of AlCl₃ at 10%, 1 mL of 1M NaOH and 1.2 mL of ultrapure water were added. The absorbances were recorded at 510 nm using a NanoPhotometer™ UV/VIS spectrometer (Implen GmbH, Germany) and flavonoids concentration was determined using a catechin calibration curve.

Quantification of lipid peroxidation

Determination of lipid peroxidation was performed through malondialdehyde (MDA) quantification, following a modified version of the protocol described by Li (2000). In brief, to 0.1 g of leaf sample were added 10 mL of 0.5% thiobarbituric acid in 20% trichloroacetic acid. Each sample was homogenised through vigorous agitation for 30 seconds and incubated in a water bath at 100 °C for 30 minutes. After the incubation period, the reaction was terminated by transferring the samples into ice. Samples were centrifuged for 10 minutes at 5.000 rpm and the supernatant was filtrated. The absorbance was measured at 450, 532 and 600 nm and MDA was quantified through the equation:

$$\text{MDA } (\mu\text{mol. L}^{-1}) = 6.45 \times (A_{532} - A_{600}) - 0.56 \times A_{450}$$

Extraction and isolation of plant-associated bacterial populations

Analysis of plant bacterial population was carried out at the end of the assay (35 days after infection), in mock-inoculated non-treated control plants (niCTR), in non-treated infected control plants (iCTR) and infected plants treated with the two different fungicides (iCO and iPP). The stems of three plants randomly selected from each treatment were separated into small portions, which were sterilized by submerging in 75% ethanol for 15 seconds, and the excess ethanol was removed by washing in deionized water (Xie and Zhao 2008). The extremities of each stem segment were removed in aseptic conditions, and each segment was cut horizontally and placed in Nutrient Agar (NA) medium with the vascular tissue facing down. After incubation at 26 °C for 3 days, morphologically distinct bacterial colonies were identified and isolated until pure cultures were obtained. For each treatment, three plants were used, and for each plant, 6 stem portions and 2 replicates were analysed.

Molecular identification of the bacterial populations

For the molecular identification of the bacterial cultures obtained as described before, the total genomic DNA of each bacterial isolate was extracted using the heat-shock method as performed by Calheiros et al.

(2010). Colonies were added to 200 µL of sterile ultra-pure water, homogenized through vigorous stirring and incubated at 95 °C for 10 minutes. Samples were then transferred into ice for 5 minutes, vortexed, and centrifuged at 15.000 rpm for 5 minutes in a microcentrifuge (Heraeus Pico 17, Thermo Scientific, USA). The concentration and integrity of the extracted DNA was evaluated spectrophotometrically using a NanoPhotometer™ UV/VIS spectrometer (Implen GmbH, Germany).

16S rRNA genes were amplified by PCR using 12.5 µL of NZYTaQ II 2x Green Master Mix (NZYTech, Portugal) with 0.5µM of primers 27F (5'-GAGTTTGATCCTGGCTCA-3') and 1493R (5'-TACCTTGTTACGACTT-3'), and 5 µL of bacterial DNA in a total volume of 25 µL. The PCR reactions were performed on a thermocycler DOPPIO (VWR, USA) using the parameters: 1 cycle of initial denaturation at 95 °C for 120s, 25 cycles of denaturation at 95 °C for 30 seconds, annealing at 54 °C for 30 seconds and extension at 72 °C for 1 minutes and finally one cycle of a final extension at 72 °C for 5 minutes. The final product was analysed by electrophoreses in a 1% agarose gel in Tris-EDTA (TAE) buffer with DNA stains Gel Red™ (Biotium, Inc., USA) for 45 minutes at 120 V and 400mA. PCR products of all 47 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).

Mineral determination by ICP-OES

For mineral determination at 28 dai, three plants were randomly selected from each treatment. Leaf samples (*ca.* 0.2 g) were mixed with 5 mL of 65% HNO₃ in a Teflon reaction vessel and heated in a Speedwave™ MWS-3p (Berghof, Germany) microwave system. The digestion procedure was conducted in five steps, consisting of different temperature and time sets: 130 °C/10 min, 160 °C/15 min, 170 °C/12 min, 100 °C/7 min, and 100 °C/3 min (Santos et al. 2015). The resulting clear solutions of the digestion procedure were then adjusted to 50 mL with ultrapure water for further analysis. Mineral determination was performed using the inductively coupled plasma optical emission spectrometer (ICP-OES) Optima 7000 DV (PerkinElmer, USA) with radial configuration. For each sample, two technical replicates were prepared.

Statistical analysis

Results were analysed using GraphPad Prism v.8 (GraphPad Software, USA). Effect of plant treatments (T) and time-point (Tp) and their interaction (T x Tp) on the number of nematodes, anthocyanin, carotenoids, chlorophyll-A and chlorophyll-B, lipid peroxidation, total soluble phenolics and flavonoids and mineral concentration in leaf tissues were analysed considering T, Tp and their interaction as fixed factors. The significant differences between the treatments with fungicides were determined using Missed-effects model (REML), which uses the restricted likelihood method and a probability value $P < 0.05$ as the threshold level of significance. The correlation between the different variables measured at 28 dai were determined using Pearson's correlation matrix.

Results

Measurement of disease symptoms and nematodes quantification

The results of the foliar damage are shown in Fig. 1. Non-infected control plants (niCTR) did not present foliar symptoms. Foliar damage was only observed in infected plants at 21 days after infection (dai), where 62.5% of the non-treated infected control plants (iCTR) presented damage on stage 1 (11–33%) and 2 (34–66%), while only 25% of infected CO-treated (iCO) and PP-treated plants (iPP) presented foliar damage of stage 1. At 28 dai, 72.5% of iCTR plants presented leaf damage, of which 12.5% were in stage 3 ($\geq 67\%$), whereas iCO only presented 62.5% of foliar damage between stage 1 and 2, and iPP presented 37.5% of foliar damage of stage 1 and 3. At the end of the assay (35 dai), 12.5% of iCTR plants had died, 72.5% presented foliar damage between the stage 3 and 2 and only 25% did not present any foliar damage. Similarly, 12.5% of iCO and iPP plants had died and 62.5% presented foliar damaged for iCO and iPP (between stage 1 and 3), and 25% did not present any foliar damage. The percentage of leaf damage was highly correlated with nematode numbers inside stem tissues (Table 3).

In inoculated plants without fungicide application, nematodes significantly increased from 1528 ± 236 (at 7 dai) to 28760 ± 3540 (at 35 dai), i.e., 18.8-fold (Fig. 2, Table 1). In contrast, in fungicide-treated plants, the number of nematodes was significantly lower in all treatments. At 35 dai the number of nematodes inside stem tissues of iCO (16667 ± 14612) and iPP (11227 ± 10307) were 0.42- and 0.61-fold significantly lower than in iCTR (28760 ± 3540).

Table 1

Effect of time-point (Tp, 7, 14, 21, 28 and 35 days after inoculation) and plant treatments (T, infected non-treated control trees (iCTR), infected trees treated with copper oxide (iCO) or potassium phosphonate (iPP)) and their interaction (T x Tp) on the number of nematodes, anthocyanin, carotenoids, chlorophyll-A and chlorophyll-B, lipid peroxidation, total soluble phenolics and flavonoids. Significant P values (< 0.05) are indicated in bold.

Response variable	Factor	Fratio	Pvalue
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 (mmol.g ⁻¹ leaf)	Treatment (T)	7.11	0.0017
	Time-point (Tp)	15.00	< 0.0001
	T ´ Tp	18.90	< 0.0001
Carotenoids (mmol.g ⁻¹ leaf)	Treatment (T)	0.63	0.5389
	Time-point (Tp)	2.75	0.0489
	T ´ Tp	7.89	< 0.0001
Chlorophyll-A (mmol.g ⁻¹ leaf)	Treatment (T)	1.35	0.2952
	Time-point (Tp)	7.03	0.0005
	T ´ Tp	2.55	0.0211
Chlorophyll-B (mmol.g ⁻¹ leaf)	Treatment (T)	1.21	0.3336
	Time-point (Tp)	1.58	0.2103
	T ´ Tp	11.16	< 0.0001
Total soluble phenolics (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

Primary and secondary metabolites

In general, both treatments (T) and time-points (Tp) were significant for anthocyanin while only time-points and the interaction T x Tp were significant for carotenoid content (Table 1). Anthocyanin (Fig. 3A) accumulation in non-treated infected control plants (iCTR) showed a progressive and significant decrease from 7 to 28 dai (by 0.43-fold), slightly increasing at 35 dai (reaching $9.80 \pm 1.94 \mu\text{mol. g}^{-1}$ leaf). In infected CO-treated (iCO), the concentration of anthocyanins gradually increased until 35 dai (by 1.32-fold), greatly increasing at 28 dai (reaching $16.82 \pm 4.72 \mu\text{mol. g}^{-1}$ leaf). At 15 dai, PP-treated plants (iPP) presented the highest anthocyanin concentrations ($37.80 \pm 10.89 \mu\text{mol. g}^{-1}$ leaf). The anthocyanin was negatively and highly correlated with nematode numbers inside stem tissues and the leaf damage (Table 3). Despite the significant influence of treatments and time-points in anthocyanins concentration in leaf tissues, no significant differences were observed in the other leaf metabolites analysed (chlorophyll-A and chlorophyll-B, Table 1 and Fig. 1S).

Both treatments and time-points significantly affected total soluble phenols and flavonoids content (Table 1). Moreover, a positive correlation was observed between soluble phenols and flavonoids content and foliar damage and nematode density, whereas flavonoids content soluble phenols were found to be negatively correlated to anthocyanins (Table 3). Non-treated infected control plants (iCTR) showed a gradual decrease in phenols and flavonoids accumulation along time (by 0.70- and 0.49-fold, respectively), but a significant increase was observed at 28 dai in phenols (by 1.65-fold) (Fig. 4). iCO showed a gradual decrease in phenols accumulation throughout the experimental period (by 0.71-fold), while flavonoid accumulation showed a gradual increase until 21 dai (by 1.39-fold), followed by a decrease until the end of the experiment (by 0.76-fold). Contrastingly, iPP showed an increasing trend in soluble phenols and flavonoids concentrations, with the highest concentration being recorded at 28 dai (by 1.49- and 2.28-fold, respectively), decreasing at 35 dai (by 0.67 and 0.40-fold, respectively) (Fig. 4).

Lipid peroxidation

In general, infected plants (both with and without fungicide treatment) displayed a progressive and significant increase in MDA levels until 28 dpi, slightly decreasing at 35 dai (Fig. 5). From 1 to 35 dpi, MDA significantly increased in all treatments (by 1.42-fold in iCTR, 1.21-fold in iCO and 1.57-fold in iPP), comparing with niCTR.

Mineral composition

Plant treatments significantly affected the concentration of micronutrients (B, Cu, Fe and Zn) and macronutrients analysed (K and P) (Table 2). The highest B concentration was found in niCTR ($36.9 \pm 3.7 \mu\text{g.g}^{-1}$), whereas iCO had the lowest ($24.1 \pm 7.2 \mu\text{g.g}^{-1}$) (Fig. 6A). The average concentrations of Cu were similar between niCTR, iCTR and iPP (around $3 \pm 0.4 \mu\text{g.g}^{-1}$), while iCO presented the highest average concentration ($189.7 \pm 39.1 \mu\text{g.g}^{-1}$; Fig. 6B). Fe concentration was higher in non-infected control plants (niCTR; $0.16 \pm 0.02 \mu\text{g.g}^{-1}$), and lowest in iCO ($0.11 \pm 0.02 \mu\text{g.g}^{-1}$ leaf; Fig. 6C). Moreover, iCTR presented the highest Zn concentration ($84.88 \pm 11.17 \mu\text{g.g}^{-1}$), while iCO had the lowest (51.46 ± 8.94

$\mu\text{g.g}^{-1}$; Fig. 6D). The infected trees (iCTR, iCO and iPP) presented a similar average concentration of K (around $7570 \pm 2183 \mu\text{g.g}^{-1}$), while niCTR presented the highest concentration ($12.2 \pm 1.3 \mu\text{g.g}^{-1}$; Fig. 6E). Finally, iCO presented the lowest concentration of P ($2.4 \pm 0.55 \mu\text{g.g}^{-1}$), while iCTR had the highest ($6.1 \pm 2.3 \mu\text{g.g}^{-1}$; Fig. 6F).

Table 2
Effect of plant treatments at 28 days after inoculation (T, non-infected non-treated control plants (niCTR), infected non-treated control trees (iCTR), infected trees treated with copper oxide (iCO) or potassium phosphonate (iPP)) on the concentration ($\mu\text{g.g}^{-1}$) 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 ($\mu\text{g.g}^{-1}$ leaf)	Treatment (T)	5.05	0.0092
	Cu ($\mu\text{g.g}^{-1}$ leaf)	Treatment (T)	136.8	< 0.0001
	Fe ($\mu\text{g.g}^{-1}$ leaf)	Treatment (T)	6.88	0.0023
	Zn ($\mu\text{g.g}^{-1}$ leaf)	Treatment (T)	7.917	0.0011
MaN	K ($\mu\text{g.g}^{-1}$ leaf)	Treatment (T)	8.06	0.0010
	P ($\mu\text{g.g}^{-1}$ leaf)	Treatment (T)	8.67	0.0007

Bacterial endophytic population

The groups with the highest bacterial diversity at the end of the experimental period were iCO with four genera and iCTR with three different genera, whereas niCTR showed two different genera and iPP only one (Fig. 7).

The main genera found were *Pseudomonas*, represented by two species, *P. fluorescens* present in all groups and *P. monteilii* (only present in iCO). The second more abundant genus found was *Klebsiella*, represented by only one specie (*K. oxycota*) present in all groups except in iPP. Other genera found was *Bacillus*, represented by only one specie (*B. cereus*) in iCTR and the genus *Pantoea*, represented by *P. agglomerans* present only in iCO.

Table 3 Person's correlation matrix between the different variables measured at 28 days after inoculation: % FDamage (percentage of foliar damage), Nnemat (number of nematodes), Antho (anthocyanins), phenols (total phenols), Flavo (total flavonoids), LPerox (lipid peroxidation), micronutrients (B, Cu, Fe and Zn) and macronutrients (K and P).

Variables	%FDamage	Nnemat	Antho	Phenols	Flavo	LPerox	B	Cu	Fe	Zn	K	P
%FDamage	1	0.936	-0.840	0.954	0.394	-0.888	0.981	-0.796	0.996	0.914	0.893	0.949
Nnemat		1	-0.977	0.998	0.693	-0.668	0.986	-0.958	0.903	0.998	0.994	0.776
Antho			1	-0.964	-0.829	0.496	-0.929	0.997	-0.792	-0.988	-0.994	-0.626
Phenols				1	0.650	-0.710	0.994	-0.940	0.926	0.993	0.986	0.811
Flavo					1	0.074	0.563	-0.870	0.315	0.733	0.766	0.083
LPerox						1	-0.783	0.428	-0.923	-0.625	-0.585	-0.988
B							1	-0.897	0.962	0.975	0.963	0.870
Cu								1	-0.742	-0.973	-0.983	-0.564
Fe									1	0.877	0.852	0.972
Zn										1	0.999	0.739
K											1	0.704
P												1

0.00 - 0.30
 0.31 - 0.50
 0.51-0.70
 0.71 - 1.00

Discussion

Fungicide application, particularly with PP, ameliorates the development of the pine wilt disease

When pine plants are infected, two infection stages take place. In the first stage, most nematodes remain close to the inoculation site and surrounding cortical tissues (Suzuki 1984). The second phase occurs after transpiration rates are impaired, and is characterized by a substantial reduction in oleoresin flow. At this stage, there is an exponential increase in PWN population and disease symptoms progression (Suzuki 1984, Myers 1988). In the current work, the increase in the number of nematodes and the beginning of the appearance of foliar symptoms occurred at 21 dai, indicating the successful infection and multiplication of the PWN inside plant tissues over time. In previous work, with one-year-old (40–50 cm height) *P. pinaster* plants maintained in a growth chamber (16 h light/8 h darkness photoperiod at 25/18°C), PWN infection progressed at higher rates between 7 and 14 days after inoculation (Nunes da Silva et al. 2021). This divergence could result from differences in plant age and size between the two experiments. Compared with non-treated plants, fungicide treatment decreased nematode density by up to 0.58- and 0.39-fold (for CO and PP, respectively) at the end of the experimental period.

Anthocyanins and carotenoids are involved in plant protection against photosystem damage (Elkhouni et al. 2018). It appears that the increase of these metabolites occurs at the beginning of the infection and the end of the assay in infected non-treated control plants (iCTR) as an effort by plants to lessen the harmful effects caused by PWN (Nunes da Silva et al. 2021, López-Villamor et al. 2022). Regarding plants treated with fungicides, iPP had a substantial peak of anthocyanin concentration at 14 dai. In comparison, iCO had a higher concentration at 28 dai, suggesting a higher antioxidant response to the PWN corroborated by the lower presence of foliar damage at that time (Bali et al. 2018, Gökbayrak and Gözel 2022, López-Villamor et al. 2022). In fact, despite preventing nematode reproduction in plant tissues, CO and PP fungicides did not prevent cellular damage, leading to increased MDA accumulation and greater foliar damage. These effects could result from the abrupt induction of plant defence mechanisms upon PWN infection (Agrawal et al., 2002). The peroxidation of unsaturated lipids in cell

membranes results from xylem parenchyma and cortex cell necrosis, and the destruction of phloem caused by PWN in susceptible species of the genus *Pinus* (Apel and Hirt 2004, Yamada 2008). The accumulation of MDA, a secondary compound of lipid peroxidation reactions, indicates cell damage induced by free radicals (Santos et al. 2012). Polyphenols, flavonoids, anthocyanins and carotenoids are also secondary metabolites in plant tolerance to biotic stress (Kawaguchi 2006, Kuroda et al. 2011, Kusumoto et al. 2014, Gaspar et al. 2017). Total soluble phenols and flavonoid concentration gradually decreased until the end of the experimental period in iCTR and iCO, increasing progressively until 28 dai in iPP. At 28 dai, polyphenol concentration correlated significantly with increased colonization by PWN (Table 3). In fact, in iCTR there was both a higher nematodes density and a more significant accumulation of phenols, as compared with plants treated with fungicides (Fig. 4A). This observation is consistent with a positive correlation between nematode migration and polyphenols concentration, previously reported in a migration assay of PWN through wood tissues of two-year-old branches from 10 years old plants (Zas et al. 2015).

Minerals play an important role in tolerance against the PWN

Mineral concentration in leaf tissues was analysed at 28 dai, coinciding with the point of most significant biotic stress indicated by the high accumulations of polyphenols and MDA (Fig. 4A and 5), increased progression of disease symptoms and higher PWN density inside plant tissues (Figs. 1 and 2).

Minerals and other nutrients have important specific functions in plant physiology, are essential for plant metabolism, and play an important role in defending plants against biotic and abiotic stress ((Waraich et al. 2011, Ahanger and Ahmad 2019, Pathak et al. 2020). Many of these minerals (Cu, Fe, Mn and Ni) are constituents of enzymes, specifically metalloproteins; others (Mn and Zn) participate in the activation of enzymes, and some (B and Zn) are constituents of plant cell walls and membranes (Asher et al. 1991, Römheld and Marschner 1991, Bergmann and Caesar 1994, Welch and Shuman 1995, Mengel and Kirkby 2001, Kirkby and Römheld 2004, Epstein and Bloom 2005, Marschner 2011). For this reason,

Fungicide application can alter the mineral composition of plant tissues (Shahid et al. 2018, dos Santos Silva et al. 2020, Motta-Romero et al. 2021). Zou et al. (2000) observed a significant relationship between the tolerance of Masson pine (*P. massoniana*) against the PWN and leaf mineral content, and this correlation changed at different stages of plant development. Likewise, differences in plant mineral content have been reported following infection by *B. xylophilus*. In the current work, there was a decrease in the concentration of B in infected plants (Fig. 7A), compared with non-infected control plants (niCTR). B is involved in carbohydrate metabolism, and when B is limited, the pentose phosphate pathway becomes predominant in carbohydrate degradation, leading to the formation of phenolic compounds (and tryptophan) through the shikimic acid pathway (Chen et al. 2014). However, the accumulation of phenols and the increased activity of polyphenol oxidase in plant tissues lead to the formation of highly reactive intermediates, such as quinones (Ruíz et al. 1998, Ölçer and Kocaçalışkan 2007). These compounds, and also photo-activated phenols, are highly effective in the production of superoxide

radicals, which may damage membranes through lipid peroxidation. Therefore, the decrease in B caused by PWN infection seems to trigger oxidative stress, causing the accumulation of polyphenols and MDA.

Cu is somewhat similar to Fe in that it forms highly stable chelates that allow the transfer of electrons (Printz et al. 2016). For this reason, it plays a role comparable to Fe in redox processes (Yruela 2005). Several Cu-containing proteins play critical roles in photosynthesis, respiration, superoxide radical detoxification, and lignification (Droppa and Horváth 1990, Burkhead et al. 2009, Broadley et al. 2012). Cu deficiency causes the accumulation of phenols (Pilon et al. 2006, Hänsch and Mendel 2009); therefore, this mineral is vital to increase plant tolerance to several diseases (Schulten and Krämer 2017). Fungicides such as copper sulphate, in which the main active ingredient is Cu, have shown *in vitro* nematicidal activity, causing high mortality and decreasing the mobility of *B. xylophilus* (Tan et al. 2013). In the current study, iCTR and iPP had the lowest Cu concentrations (Fig. 6B). The high negative correlation with foliar damage and the number of nematodes and the high positive correlation with flavonoids (Table 3) found in this trial supports that the lower Cu concentration may be related to the higher foliar damage (Fig. 1C). Accordingly, iCO presented higher concentrations of Cu (Fig. 6B) and lower foliar damage (Fig. 1C).

Cytochrome oxidase, catalase, and peroxidase are Fe-dependent enzymes (Hänsch and Mendel 2009), and their activities often decrease under conditions of Fe deficiency (Mengel and Kirkby 2001). A drastic decrease in peroxidase activity with subsequent accumulation of phenolic substances has been reported under Fe-limiting conditions (Römheld and Marschner 1981). Here, the lowest concentrations of Fe (Fig. 6C) were observed in infected plants when compared to the non-infected control plants (niCTR), and the former were also the ones with the highest accumulation of phenols and lipid peroxidation (Table 3). This suggests that Fe plays a vital role in the regulation of enzymes that could prevent the oxidation of tissues caused by PWN.

Zn has an essential role in plant metabolism, including effects on carbohydrate metabolism, protein synthesis, hormonal regulation, and membrane integrity (Mengel and Kirkby 2001). Here we found a high correlation (Table 3) between higher Zn levels in non-treated infected control plants (iCTR) (Fig. 7C), which also had higher concentrations of phenolic compounds (Fig. 4A) and nematodes numbers (Fig. 2). In *Kandelia obovata* a strong correlation was also found between Zn concentrations and the accumulation of phenolic compounds (Chen et al. 2019). On the other hand, Georgieva et al. (2002) suggested that plants grown at higher Zn levels have higher nematode levels, but this was probably due to adverse effects on nematode antagonists. Therefore, the increase in Zn may be related to the higher number of PWNs isolated in the non-treated infected control plants (iCTR) as opposed to the lower concentration of this micronutrient and, therefore, a lower number of nematodes observed in fungicide-treated plants.

Potassium (K) plays an essential role in plant metabolism, supporting plants' fitness under biotic and abiotic stresses (Wang et al. 2013). K-deficient plants generally are more susceptible to biotic diseases than plants with an adequate K supply (Mukherjee et al. 2019). In the present assay, non-infected control

plants (niCTR) had the highest concentrations of K (Fig. 6E), when compared to the infected plants, which were the ones with the highest accumulation of phenols and lipid peroxidation (Table 3), corroborating that K plays an essential role in defence regulation following PWN infection. Manghi et al. (2021) showed that plants could not use PP as a source of K and P. Hence its concentration does not increase in the analysed tissues (Manghi et al. 2021).

Phosphorus (P) plays a vital role in plant immunity and is one of the most relevant minerals related to plant growth (Chan et al. 2021). The presence of high concentrations of Cu and PP negatively influences P absorption (Rawat et al. 2018, Manghi et al. 2021). In addition, P deprivation induces the synthesis of secondary metabolites with antimicrobial activity, such as flavonoids and glucosinolates (Pant et al. 2015), and defence hormones, such as salicylic acid (SA) and especially jasmonic acid (JA) (Khan et al. 2016, Castrillo et al. 2017, Morcillo et al. 2020), which modify the immunity of plants. Therefore, the low levels of this mineral observed in iCO and iPP (Fig. 6F), compared to the high levels in iCTR, along with the lower levels of damage and nematode density (Figs. 1 and 2) observed in fungicide-treated plants, might result from the induction of defence hormones caused by P limitation.

Fungicide treatment induces changes in the bacterial community of *P. pinaster*

La Torre et al. (2018) demonstrated that CO can combat bacterial diseases and that PP activates different plant defence pathways, SAR and LAR (INTAGRI 2017). These pathways produce exudates that can alter the plant microbiome (Lebeis et al. 2015, Liu et al. 2017, 2020, Mannaa et al. 2020). Since PWD-associated endophytic bacteria may be necessary to develop or suppress infection caused by the nematode (Alves et al. 2018, Kim et al. 2019), the present work aimed to study the modifications of PWN-associated bacterial communities after fungicide treatment. We observed that the non-infected control plants had two species, *K. oxytoca* and *P. fluorescens*. *K. oxytoca* was present in all treatments except in the plants treated with PP, while all plants had *P. fluorescens*. *K. oxytoca* is an endophytic plant growth-promoting strain (Hallmann et al. 1997); this nitrogen-fixing bacterium exists in rice roots (Nguyen T et al. 1989). Roriz et al. (2011) suggested that it may be associated with the Portuguese region because previous works did not identify this bacterium in *P. pinaster*. On the other hand, *P. fluorescens*, a plant growth-promoting rhizobacteria (PGPR), increases primary productivity by promoting growth and triggering induced systemic tolerance in plants (Hol et al. 2013, Panpatte et al. 2016). Apart from *P. fluorescens*, niCTR also presented *Bacillus cereus*, previously described in Masson pine, by having a nematocidal activity (Li et al. 2020). iCO plants had *Pseudomonas monteilii*, present in the *Pseudomonas putida* group (Anzai et al. 2000), described to promote plant growth and induce systemic tolerance to root rot fungi. *P. putida* can induce systemic resistance against PWN in seedlings and pine callus (Pandya et al. 2014, Kim et al. 2019, Urooj et al. 2020). iCO also presented *Pantoea agglomerans*, a species belonging to the family Erwiniaceae and adapted to live epiphytically in several plant tissues (Zhang and Qiu 2015, Luziatelli, Gatti, et al. 2020). Some strains of this species are agronomically important for their biocontrol activity (Dutkiewicz et al. 2016) and their ability to produce the auxin indole-3-acetic acid (Luziatelli, Ficca, et al. 2020). Although all these genera have been previously described as related to both

pine plants and the PWN (Proença et al. 2010, 2017, Roriz et al. 2011, Vicente et al. 2011), this work identified some species for the first time, such as *B. cereus* (niCTR), *P. montielli* and *P. agglomerans* (iCO). Christian et al. (2016) showed that fungicides modulate bacterial types and diversity in white snakeroot (*Ageratina altissima*), but it is the first report in pine. Therefore, the application of fungicides modifies the diversity of the *P. pinaster* microbiome favouring the proliferation of species that improve resistance against the PWN, particularly in plants treated with CO, which presented critical bacterial species related to plant growth promotion (*K. oxytoca* and *P. fluorescens*) and resistance to diseases, including the PWD (*P. agglomerans* and *P. montielli*).

Conclusions

This study describes for the first time several physiological and microbial changes occurring in *P. pinaster* plants infected with *B. xylophilus* after exogenous application of CO and PP. All infected plants (treated and no-treated) presented the same percentage of dead plants (12,5%) at the end of the assay, but fungicide-treated plants had fewer nematodes and foliar symptoms than infected control plants, thus decreasing the progression of PWD. Fungicides promoted defence mechanisms of pine plants against the PWN by increasing the concentrations of anthocyanins, carotenoids, and flavonoids in plant tissues at specific stages following infection. Infected plants treated with CO and PP had high MDA concentrations, which may correlate with a stronger redox activity. Moreover, fungicide application induced changes in the plant bacterial community, promoting beneficial bacteria for the defence of *P. pinaster* against the PWN. It also altered the concentration of several essential minerals, such as Cu and P, associated with plant growth, defence and immunity. This integrated study supports the use of Cu and P-based fungicides as a biocontrol method against PWN, for example, nursery conditions, due to their beneficial role in the accumulation of crucial plant metabolites and the promotion of plant defences.

Declarations

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Conflict of interest

The authors declare that they have no conflict of interest.

Author contribution statement

ALV and MV: designed the experiment; ALV and MN: infected the plants; ALV: developing the experiment, treated the plants with CO and PP, did the sampling, performed the biochemical, microbiological, mineral analysis and data analysis; ALV: coordinated the final writing and all authors made helpful suggestions; MV: obtained the funding, helped plan the experiments and revised the manuscript. All authors contributed to the writing and discussion of the manuscript.

Data Availability

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

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Figures

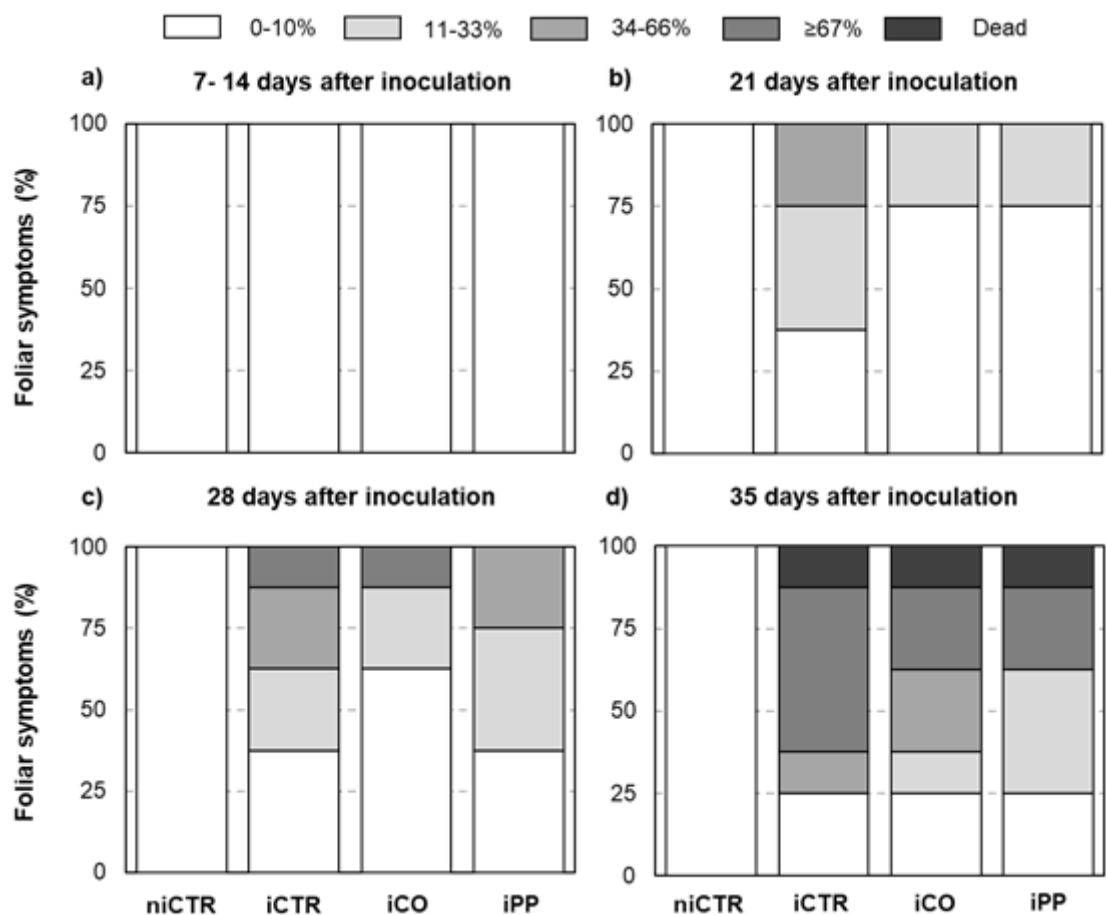


Figure 1

Foliar symptoms (%) at (a) 7-14 days, (b) 21 days, (c) 28 days and (d) 35 days after inoculation in mock-inoculated non-treated control plants (niCTR), infected non-treated control trees (iCTR), and infected trees treated with copper oxide (iCO) or potassium phosphonate (iPP)

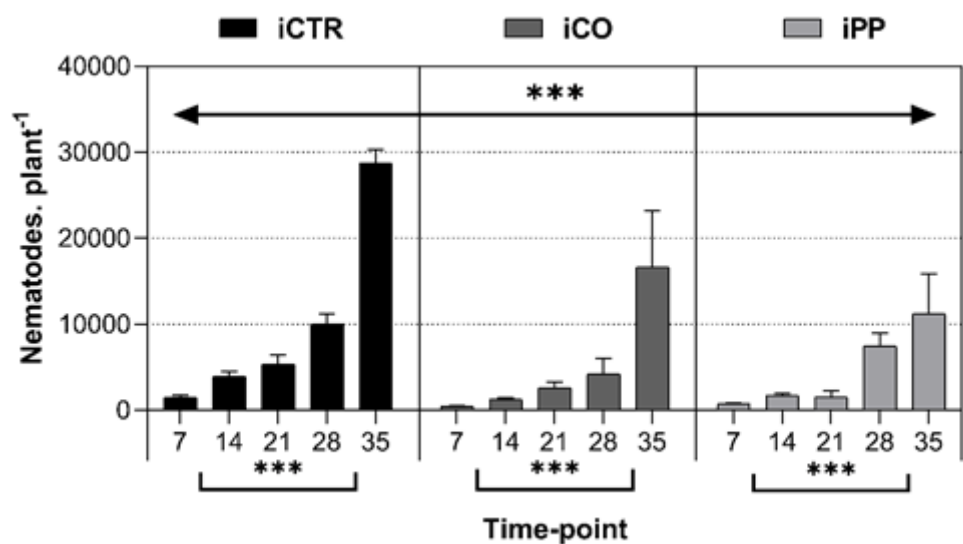
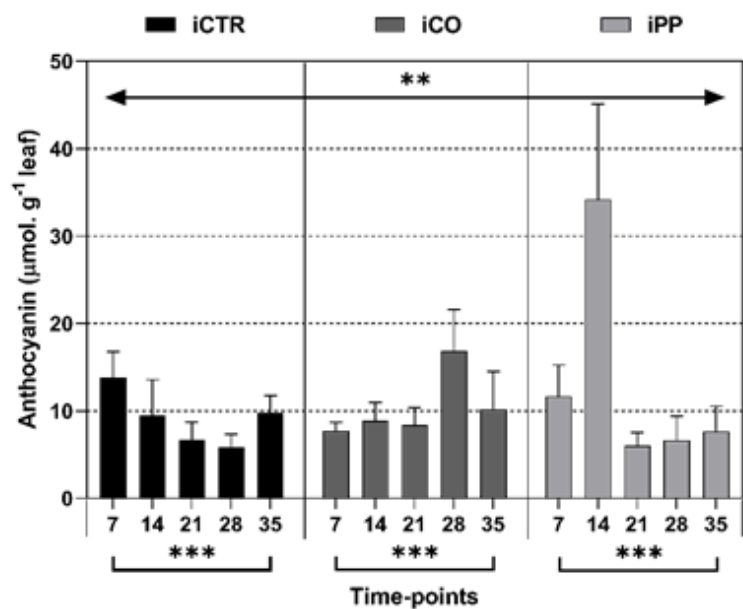


Figure 2

Number of nematodes (Nematodes.plant⁻¹) in infected non-treated control trees (iCTR), and infected trees treated with copper oxide (iCO) or potassium phosphonate (iPP) (at 7, 14, 21, 28 and 35 days after inoculation). Values represent the mean of 5 biological replicates $\pm$ standard error of the mean. Significance levels of treatments and time-point for number of nematodes: ***. $P < 0.001$; **. $P < 0.01$; *. $P < 0.05$; ns. not significant

a)



b)

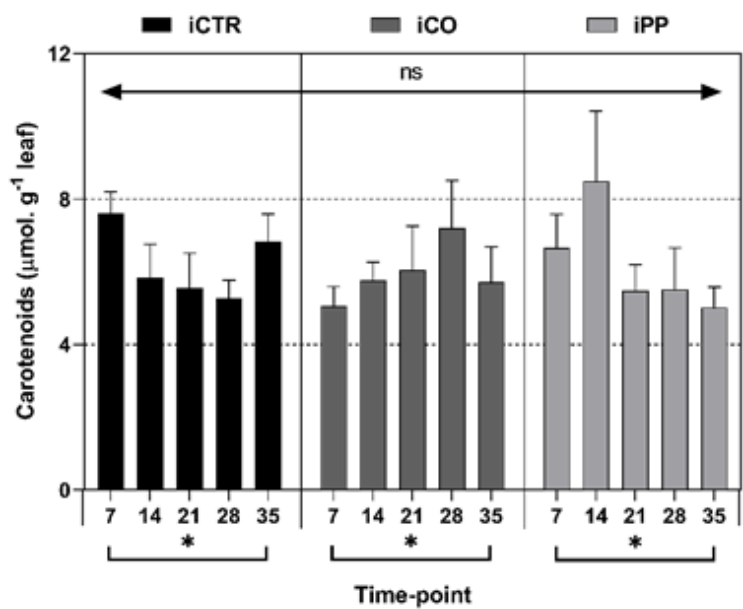
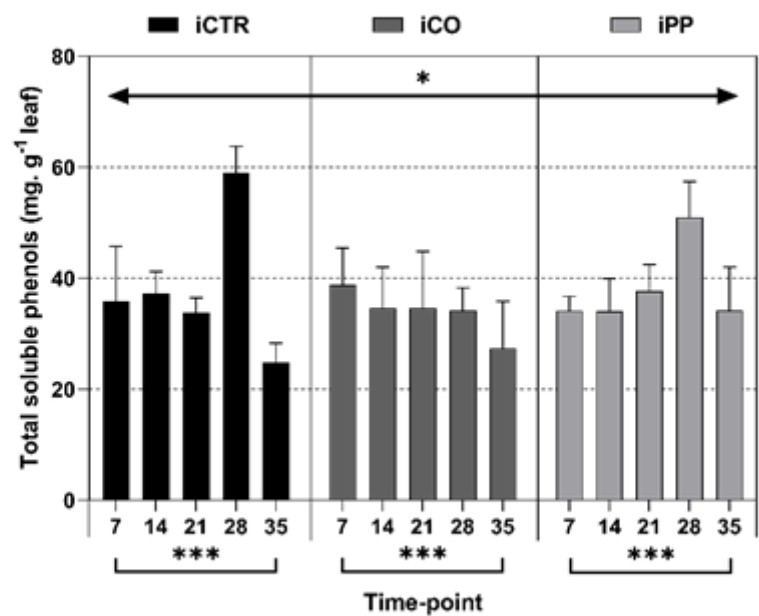


Figure 3

(a) Anthocyanin ($\mu\text{mol.g}^{-1}$ leaf tissue) and (b) carotenoids ($\mu\text{mol.g}^{-1}$ leaf tissue) in infected non-treated control trees (iCTR), and infected trees treated with copper oxide (iCO) or potassium phosphonate (iPP) (at 7, 14, 21, 28 and 35 days after inoculation). Values represent the mean of 5 biological replicates $\pm$ standard error of the mean. Significance levels of treatments and time-point for anthocyanin and carotenoids: ***. $P < 0.001$; **. $P < 0.01$; *. $P < 0.05$; ns. not significant

a)



b)

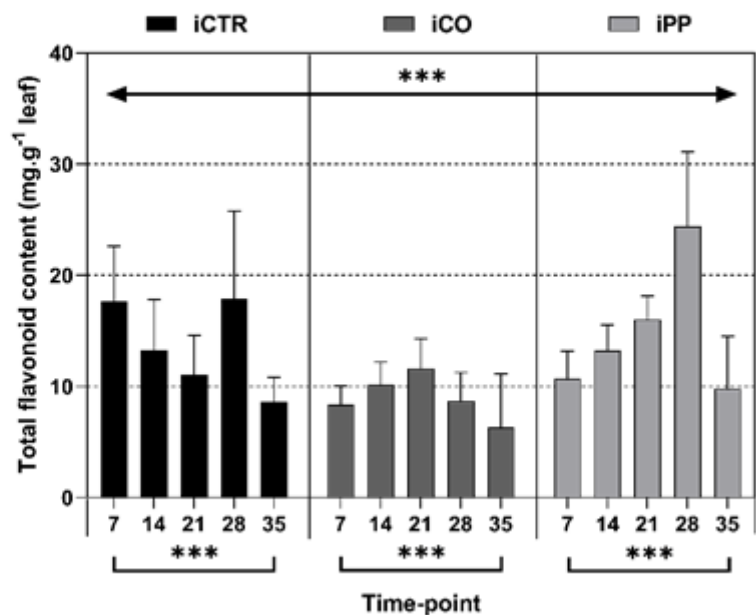


Figure 4

(a) Total soluble phenols (mg.g⁻¹ leaf) and (b) total flavonoids content (mg.g⁻¹ leaf) in infected non-treated control trees (iCTR), and Infected trees treated with copper oxide (iCO) or potassium phosphonate (iPP) (at 7, 14, 21, 28 and 35 days after inoculation). Values represent the mean of 5 biological replicates $\pm$ standard error of the mean. Significance levels of treatments and time-point for phenols and flavonoids: ***. P < 0.001; **. P < 0.01; *. P < 0.05; ns. not significant

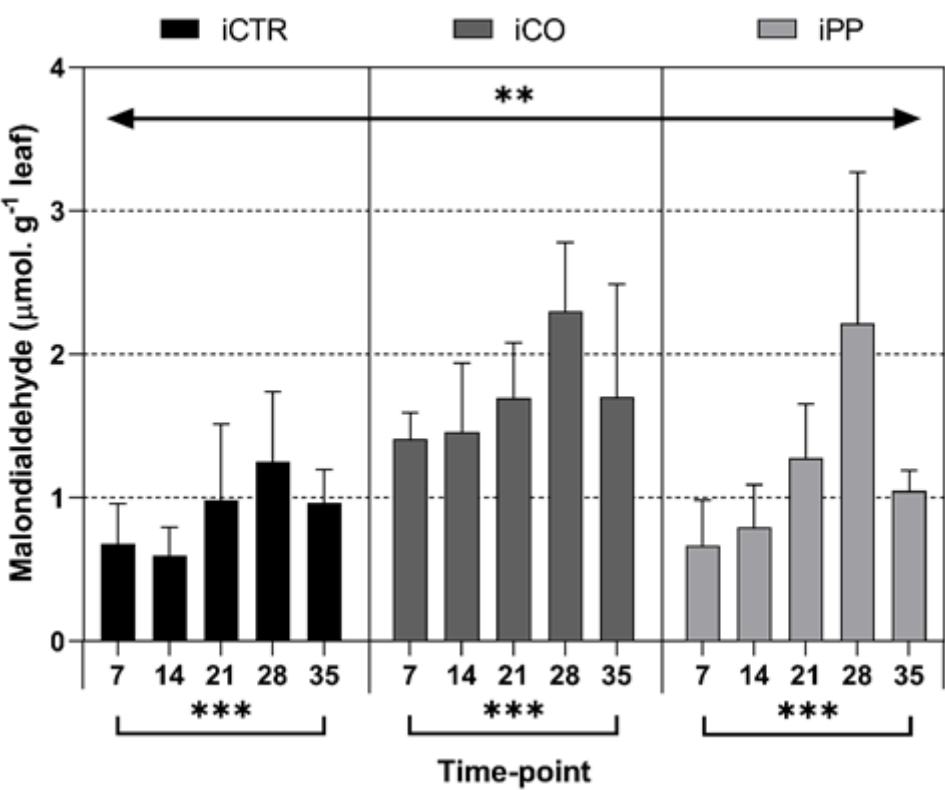


Figure 5

Malondialdehyde (MDA) (μmol.g⁻¹) in infected non-treated control trees (iCTR), and infected trees treated with copper oxide (iCO) or potassium phosphonate (iPP) (at 7, 14, 21, 28 and 35 days after inoculation). Values represent the mean of 5 biological replicates $\pm$ standard error of the mean. Significance levels of treatments and time-point for MDA: ***. P < 0.001; **. P < 0.01; *. P < 0.05; ns. not significant

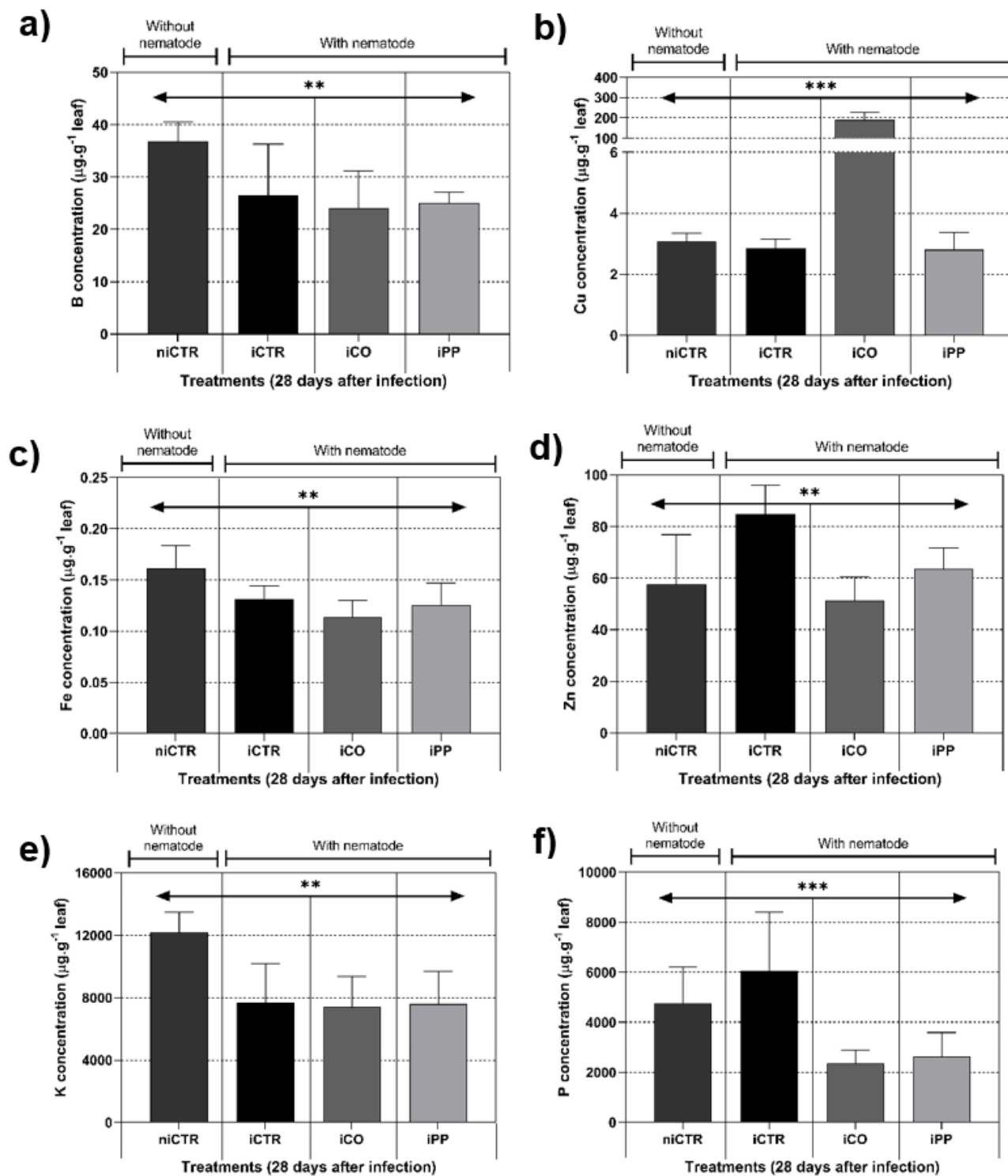


Figure 6

Concentration of the boron (B), copper (Cu), iron (Fe), zinc (Zn) micronutrients (**a-d**) and of potassium (K) and phosphorus (P) macronutrients (**e-f**) in mock-inoculated non-treated control plants (niCTR), infected non-treated control trees (iCTR), and infected trees treated with copper oxide (iCO) or potassium phosphonate (iPP) at 28 days after infection. Values represent the mean of 5 biological replicates $\pm$

standard error of the mean. Significance levels of treatments and time-point for B. Cu. Fe. Zn. K and P:
 ***. $P < 0.001$; **. $P < 0.01$; *. $P < 0.05$; ns. not significant

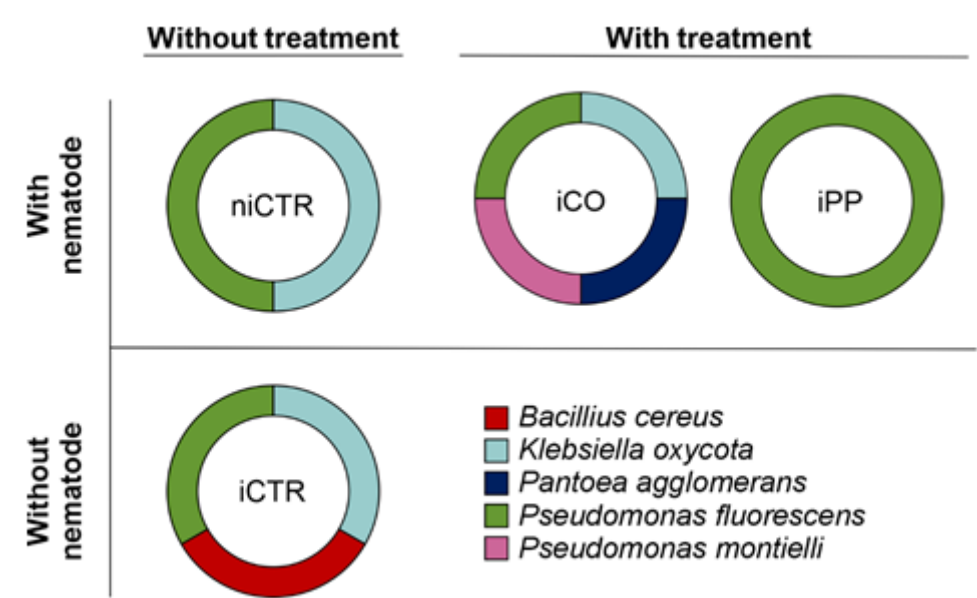


Figure 7

Bacterial populations in mock-inoculated non-treated control plants (niCTR), infected non-treated control trees (iCTR), infected trees treated with copper oxide (iCO) or potassium phosphonate (iPP) at 35 days after inoculation

Supplementary Files

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