Metalaxyl-M, phosphorous acid and potassium silicate applied as soil drenches show different chestnut seedling performance and protection against *Phytophthora* root rot

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Abstract The application as soil drenches of three commercial products containing metalaxyl, phosphorous acid or potassium silicate, were studied as a means of controlling *Phytophthora* in chestnut (*Castanea sativa* Mill.) seedlings. In the metalaxyl treatment no plant deaths were recorded, whereas with the phosphorous acid and potassium silicate applications, and in the untreated control, the mortality rate was respectively 33.3, 44.4 and 77.8%. The presence of *Phytophthora* was detected in plants and soils at the end of the experiment which means that the three products did not eliminate the pathogen, and that they only gave temporary protection to the plant. In the treatments receiving metalaxyl or phosphorous acid, the plants showed a sharp drop in growth compared to the surviving plants of the control. Soil analyses revealed a high increase in exchangeable acidity, a high reduction in pH and a high increase in Mn levels in the soils treated with the products containing metalaxyl or phosphorous acid. In these treatments, elemental tissue analysis and nutrient recovery by plants revealed Mn levels far above the upper limit of the sufficiency range, with the toxicity of Mn being the suspected cause for the strong reduction in plant growth. These results indicate that when applying such products to the soil, their concentration and/or their ability to influence the soil pH should be evaluated and adequate measures of pH adjustment undertaken. In the case of phosphorous acid, its use can be replaced by phosphite salts. K-silicate did not show adverse effects on plant growth but provided less protection against *Phytophthora* than metalaxyl or phosphorous acid.

Keywords *Castanea sativa* · Ink disease, biostimulants · Phosphite · Phosphonate · Molecular identification · Plant protection

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Introduction

In Portugal, although the planted area of chestnut trees (*Castanea sativa* Mill.) has been increasing in recent years, the production of chestnut fruit has not followed a similar trend (FAOSTAT, 2020). A set of phytosanitary problems, of which ink disease is the most prevalent, has been affecting the species, leading to the death of mature trees so that producers are constantly replacing them with less productive young trees (Martins et al., 2007; Arrobas, Afonso, & Rodrigues, 2018a; Rodrigues et al., 2019). However, this is not a local problem, with statistics from many other parts of the world, including European countries, showing the same pattern (FAOSTAT, 2020).

It was well-known from the beginning of the twentieth century that ink disease in chestnut is associated with species of the genus *Phytophthora*, namely *P. cinnamomi* Rands and *P. cambivora* (Petri) Buisman (Crandall et al., 1945; Petri, 1917). *P. cambivora* was recently re-described without nomenclatural act by Jung, Jung, Scanu, et al. (2017a) as *P. xcambivora*. Other species of *Phytophthora*, such as *P. cactorum* and *P. plurivora* (formely *P. citricola*) may also damage chestnut trees, although with a lesser degree of aggressiveness (Akilli et al., 2012; Biocca et al., 1993; Vettraino et al., 2005). In Portugal, *P. cinnamomi* is the most frequently isolated species from the chestnut ecosystem and is, therefore, considered the predominant species in the development of ink disease (Carneiro-Carvalho et al., 2019; Fernandes, 1979; Gouveia, 2004). However, Jung, Jung, Cacciola, et al. (2017b) described a new cryptic species of *Phytophthora* (*P. castanetorum*) for Portugal, in The Algarve and in the Natural Park of Serra da Estrela that can cause severe ink disease symptoms and may occur simultaneously with the predominant abovementioned species. The pathogen infects the root system, causing blackening of the thinner roots due to the decomposition of the cortex, becoming moist and rotten. Thicker roots are also attacked, showing blackened spots, due to changes in the cortex and cambium. Symptoms of the disease on the aerial part of mature trees include chlorotic leaves reduced in size, thinning of the crown and immature husks which remain on the tree after leaf-fall (Vannini & Vettraino, 2001). In young chestnuts, the entire plant acquires a yellowish appearance that quickly develops into necrosis and desiccation (Gouveia, 1993). As the result of the infection of the root system, a reduction in

water and mineral uptake occurs, which causes a detrimental effect on growth and yield and ultimately leads to the death of whole trees (Erwin & Ribeiro, 1996).

Metalaxyl, phosphites (salts of phosphorous acid) and phosphonates (phosphite esters) have been the most commonly used substances for the chemical control of *Phytophthora*, both in chestnut (Cohen & Coffey, 1986; Guest et al., 1995; Stein & Kirk, 2002) and in several other fruit tree species (Akinsanmi & Drenth, 2013; Masikane et al., 2020; Nyoni et al., 2019; Türkölmez & Derviş, 2017). Potassium silicate has become increasingly important in agriculture as a biostimulant capable of helping plants to cope with abiotic and biotic stresses (Carneiro-Carvalho et al., 2020; Savvas & Ntatsi, 2015) and is now recognized as a useful tool to be used against *Phytophthora* (Bekker et al., 2014).

Metalaxyl has long been considered the most effective and versatile fungicide for the control of diseases caused by *Phytophthora* (Schwinn et al., 1977; Urech et al., 1977). It is a systemic fungicide, with apoplastic translocation, interfering with the RNA synthesis of *Phytophthora* species. It is usually applied preventively although it can also be applied as a therapeutic fungicide on plants which are already infected (Davidse, 1987; Erwin & Ribeiro, 1996). In crops in which *Phytophthora* species attack the shoots, it is usually applied as a foliar spray (Liljeroth et al., 2016; Lu et al., 2018). In tree species in which oomycetes of the genus *Phytophthora* attack the roots, treatments such as soil drenches are also common (Akinsanmi & Drenth, 2013; Türkölmez and Derviş 2017).

Phosphite is translocated in both upward and downward directions in xylem and phloem (Erwin & Ribeiro, 1996) which allows it to be applied as foliar and trunk sprays, trunk injections or as soil drenches (Akinsanmi & Drenth, 2013; Coelho, 2009; Masikane et al., 2020). Phosphite has good properties as an inhibitor of plant pathogens (Fenn & Coffey, 1984; Gomez-Merino & Trejo-Telle, 2015), and has given good results in *Phytophthora* control in tree species in which the oomycete infects the roots (Akinsanmi & Drenth, 2013; González et al., 2020; Nyoni et al., 2019). Phosphite can control diseases caused by *P. cinnamomi*, directly through the inhibition of pathogen growth, or indirectly through enhancement of plant defences (Hardham & Blakman, 2018).

However, neither metalaxyl nor phosphites can overcome effectively the problem of ink disease in the long-term. In previous studies it has been reported that the

continued use of metalaxyl or phosphites can lead to decreased efficacy, or the development of resistance by the pathogen (Dobrowolski et al., 2008; Hu et al., 2010). Researchers are currently therefore focused on developing alternative tools to help farmers to manage this important plant disease (Ramírez-Gil and Morales-Osorio (2020).

Potassium silicate appeared on the market as a slow-release fertilizer in 1978 (Ma & Takahashi, 2002). Although the effectiveness of silicon (Si) in the control of plant diseases, as well as its mode of action, are not very well understood. Carneiro-Carvalho et al. (2017) reported that Si appears to be a possible inducer of resistance to *P. cinnamomi* in chestnut trees. Previous in vitro studies, performed by Kaiser et al. (2005), also found that 5 ml soluble Si per litre of agar inhibited the growth of *P. cinnamomi* by 100%. Thus, it seems that potassium silicate can be one more tool to manage ink disease. It is included in the group of biostimulants, in accordance with the European Union Regulation (EU) 2019/1009.

In this study, two commercial products containing active ingredients with a recognized high potential to control or reduce the incidence of ink disease, mainly metalaxyl-M and phosphorous acid, were compared with potassium silicate, a plant biostimulant of some interest but little studied in the control of *Phytophthora* in chestnut. All products were applied as soil drenches to young nursery plants. The experiment also included an untreated control. In monitoring the effects of the treatments, the growth of the seedlings, their survival rates, the elemental composition of plant tissues (roots, stems and leaves) and soil properties were assessed, and *Phytophthora* was detected and identified from soil samples.

Materials and methods

Greenhouse assay

Planting stock was obtained by stratification of seeds from certified sites of *C. sativa* in moist sand during winter to allow a better and concentrated germination. In late winter, the sprouted chestnuts were planted in pots of 4 L in volume. The growing medium was soil heavily infected (in the initial bait test of the soil almost all baits were infected with *Phytophthora*), collected from beneath the canopy of chestnut trees with severe ink disease symptoms, mixed with perlite in a ratio of 3:1 (v/v).

The experiment was arranged as a completely randomized design with four treatments and nine replications (nine plants per treatment). The treatments consisted of three commercial products, containing metalaxyl-M, phosphorous acid or potassium silicate (K-silicate), and an untreated control. The composition of the commercial product containing metalaxyl-M was 40% folpet (w/w) and 5% metalaxyl-M (w/w). It was used at a rate of 2 g L^{-1}. The composition of the product containing phosphorous acid (H_3O_3) (the conjugate acid of the phosphite anion, $H_2PO_3^-$) was 3.0% N, 35% H_3PO_3, 0.2% Fe, 2.8% Mn and 1.0% Zn (it was prepared from algae extracts, micronutrients, H_3PO_3 and coadjutants) and was applied at the concentration of 4 ml L^{-1}. K-silicate was composed of 25 to 27% SiO_2 and 12 to 13% K_2O and was used at the same concentration as phosphorous acid.

The products were applied twice, the first time on May 7th, 2018, with the second application being performed 3 weeks later. All the solutions containing the active ingredients were prepared, and 500 ml poured directly onto the soil of the pots around each plant. The control treatment received 500 ml of water.

After the installation of the experiment, the pots were weeded as required. 500 ml water was used for each irrigation. The number of weekly watering's depended on the environmental conditions in the greenhouse. The irrigation frequency was adjusted in order to avoid exceeding the field capacity and anaerobic conditions of the growing medium.

Plant performance evaluation and tissue and soil sample collection

Plant death was recorded throughout the experimental period. At the end of the experiment (October 29th, 2018), several morphological traits, such as plant height, stem diameter and the length and width of the leaves were measured. Thereafter, the live plants were uprooted and separated into roots, stems and leaves. The roots were washed with a gentle flow of water. From each treatment, samples of blackening and rotten roots were taken for detection of *Phytophthora*. All plant tissues were oven-dried at 70 °C, weighed and ground. Soil samples were also taken after the soil in each pot was properly mixed. Two subsamples were

prepared, the first for the detection of *Phytophthora* and the other for the determination of the effect of the treatments on soil properties. For detection of *Phytophthora*, the soil samples did not receive additional pre-treatments; they were used fresh as collected from the pots. For the determination of soil properties, the soil was oven-dried at 40 °C.

Detection and identification of *Phytophthora* species

For *Phytophthora* detection in the soil samples, the method of biological trap with chestnut leaf discs, as proposed by Gouveia et al. (2009), was used. From each sample, 20 ml of soil was placed on a 120 mm diameter Petri dish. In each dish, 8 ml of distilled water was added along with twenty disks of chestnut leaves, 0.5 cm in diameter. The dishes were placed at room temperature and, after 24, 48 and 72 h, three discs of leaves from each dish were removed and placed in PARPH selective culture medium (Pimaricin, 5 mg L^{-1}; Ampicillin, 250 mg L^{-1}; Rifanpicin, 10 mg L^{-1}; Hymexazole, 25 mg L^{-1}) for *Phytophthora* growth. The same PARPH selective culture medium was used at the end of the experiment for detection of *Phytophthora* from roots with symptoms of blackening and moist decay. The presence of *Phytophthora* was confirmed by microscopic observation at the hyphae level. After mycelial growth, the isolates were cultured on PDA medium (Potato Dextrose Agar, Difco 39 g L^{-1}) for later molecular identification of the species.

The identification of *Phytophthora* species was based on morphological characteristics and molecular methods by amplification and sequencing of the ITS (ITS1, 5.8S, ITS2) in the ribosomal DNA (rDNA) region using the ITS1 and ITS6 universal primers (White et al., 1990) and comparing with sequences published in the GenBank databases, BLAST (https://blast.ncbi.nlm.nih.gov/). DNA extraction from *Phytophthora* isolates was performed using the REDExtract-N-Amp™ Plant PCR Kit (Sigma-Aldrich), and amplified region sequencing was performed by Stabvida Laboratories (Caparica, Portugal). Each species was classified taxonomically according to the MycoBank Database (www.micobank.org).

Soil and plant tissue analyses

Soil samples were submitted to the following analytical determinations: 1) pH (H_2O and KCl) (by

potentiometry); 2) organic C (Walkley-Black method); 3) cation exchange capacity (ammonium acetate, pH 7.0); 4) extractable P and K (ammonium lactate solution at pH 3.7); 5) extractable Fe, Mn, Zn and Cu (ammonium acetate and EDTA, determined by atomic absorption spectrometry); and 6) extractable B (Hot water, and azomethine-H). Methods 1–3, 5 and 6 were fully described by Van Reeuwijk (2002) and method 4 by Balbino (1968).

Elemental analyses of all the tissues (leaves, stems and roots) were performed by Kjeldahl (N), colorimetry (B and P), and atomic absorption spectrophotometry (K, Ca, Mg, Fe, Mn, Cu, Zn) methods (Temminghoff & Houba, 2004) after tissue samples were digested with nitric acid in a microwave.

Data analysis

Data was firstly tested for normality and homogeneity of variances using the Shapiro-Wilk test and Bartlett's test, respectively. The comparison of the effect of the treatments was provided by one-way ANOVA. When significant differences were found ($\alpha < 0.05$), the means were separated by the multiple range Tukey HSD test ($\alpha = 0.05$).

Results

Plant growth

All the chemical treatments reduced the rate of plant mortality in comparison to the control. In the metalaxyl treatment all the plants survived, whereas in the control treatment only 22.2% survived (Table 1). However, at the end of the experiment, surviving plants of the control treatment showed an increased average height, stem diameter, leaf number and a higher number of fully developed leaves, although significant differences between treatments ($P < 0.05$) were only found in stem diameter and leaf number.

The surviving plants in the control treatment showed significantly higher dry matter yield in comparison to the plants of the phosphorus acid and metalaxyl treatments (Fig. 1). This extended to the dry biomass of roots, stems and leaves. The plants of the K-silicate treatment showed total dry matter yield not significantly different from that of the control treatment.

Detection and identification of *Phytophthora*

The bait method for soil detection of *Phytophthora* with chestnut leaf discs revealed the presence of *Phytophthora* hyphae in the control treatment after 24 h of contact between the soil and the chestnut leaves (Table 2). In the treated plants, the presence of hyphae of the oomycete only occurred after 48 h of incubation. At 48 and 72 h of incubation all the treatments including metalaxyl revealed the presence of *Phytophthora* hyphae in the baits.

From the isolates obtained from the discs of chestnut leaves infected with *Phytophthora*, 11 isolates were selected for molecular identification of the species. The sequences were compared with other sequences published in the GenBank databases using Blast and Blastn (https://blast.ncbi.nlm.nih.gov/). The sequence identities of 10 isolates of *Phytophthora* were above a 99% cut-off to the ex-type isolate (NCBI Accession number: FJ801806) used to compare sequences obtained in this study. For one isolate obtained in the control (pot 2) the sequence identity was 98.00%. However, as in the Blast, the obtained results showed that all 100 sequences selected belonged to the species *P. cinnamomi*, so we consider that by the Blast criteria this isolate is *P. cinnamomi* (Table 3).

P. cinnamomi was also detected from root and collar rot lesion samples in each treatment including the metalaxyl-M treatment. After molecular identification, sequences were deposited in the NCBI data base with the accession number: MW769784, MW769927, MN960436, MW772238, MW772384, MW769778 and MW772365. Sequence based identification of *Phytophthora* obtained from the root tissue of plants revealed the same percentage of base identity obtained from the soil, including pot 2 of the control, with values close to 98%.

Tissue elemental composition and soil properties

Root N concentrations varied significantly between treatments (Table 4). The plants of the phosphorous acid treatment showed significantly higher values than those of the other treatments. The plants of the metalaxyl treatment, in turn, showed significantly higher leaf N concentrations than that of the control and K-silicate treatments. For concentrations of P in the roots, the values of the plants treated with phosphorous acid stand out with values significantly higher than that of the other

treatments. Root K concentrations were significantly higher in the plants of the K-silicate in comparison to that of the other treatments. Ca concentrations in the roots did not vary significantly between treatments. The higher values of Mg in the roots were recorded in the phosphorous acid and metalaxyl treatments, whereas the higher B and Zn values were found in the phosphorous acid, and the higher Mn values in the phosphorous acid followed by metalaxyl treatments.

Leaf N concentration was significantly higher in the metalaxyl and phosphorous acid treatments than in the K-silicate and control treatments (Table 5). Leaf P levels, in turn, were significantly higher in the phosphorus acid in comparison to the other treatments. Leaf K levels were significantly higher in the K-silicate treatment in comparison to the others. The values of Ca and Mg in the leaves reached the highest and lowest values, respectively, in the metalaxyl and K-silicate treatments. B values also registered significant differences between treatments, with the higher values to be found in the phosphorous acid and metalaxyl treatments. In the cases of Cu and Fe, the higher values were recorded respectively in the metalaxyl treatments and the control. The values of Zn and Mn were markedly higher in the phosphorus acid treatment.

The concentrations of nutrients in the stems followed the general pattern observed in the leaves (data not shown), although they were lower in relative terms for all nutrients, showing a tissue with greater lignification and C concentration.

Nutrient recovery is the product of dry matter yield and tissue nutrient concentration. N recovery in total plant biomass did not vary significantly between treatments. P recovery was significantly higher in the phosphorus acid in comparison to metalaxyl treatment (Table 6). K recovery was significant and particularly higher in the K-silicate in comparison to the other treatments. The recovery of Ca, Mg and B in plant tissues did not vary significantly between treatments. The higher recovery values of Cu and Fe were found in the K-silicate treatment, whereas the higher values of Zn and Mn were observed in the phosphorous acid treatment.

Soil organic C did not change significantly with fungicidal treatments, varying between 23.8 to 27.8 g kg^{-1} (Table 7). However, the treatments influenced soil pH greatly. The values decreased significantly in the order of K-silicate (6.3), control (5.6), metalaxyl (4.9) and phosphorous acid (4.4). Extractable

Table 1 Morphological traits of surviving plants (average ± standard deviation) at the end of the experimental period

	Live plants (%)	Plant height (cm)	Stem diameter (cm)	Leaf number	Fully developed leaves (%)
Control	22.2	74.5±51.62 a	1.3±0.14 a	21.0±6.00 a	72.2±5.56 a
Phosphorous acid	66.7	25.9±15.05 a	0.6±0.23 b	16.2±4.07 b	40.5±15.78 a
Metalaxyl	100	24.2±20.00 a	0.7±0.19 b	12.8±3.96 b	41.0±14.55 a
K-silicate	55.6	34.0±27.38 a	0.9±0.34 ab	17.6±5.13 ab	51.7±15.06 a
Prob.		0.0807	0.0093	0.1052	0.0587

Means followed by the same letter are not significantly different by Tukey HSD test ($\alpha = 0.05$)

P decreased significantly from the phosphorus acid (174.1 mg kg^{-1}) to the control treatment (105.7 mg kg^{-1}). Extractable and exchangeable K were approximately six times higher in the K-silicate than in the other treatments. Exchangeable Ca and Mg were also the highest in the K-silicate treatment. Exchangeable acidity was higher in the soil of the pots receiving phosphorous acid, which were also those showing the lower pH values. Extractable Zn was particularly high in the phosphorous acid pots, whose average value was 21.4 mg kg^{-1} whereas, in the other pots, Zn values varied from 1.3 to 1.6 mg kg^{-1}. Phosphorous acid and K-silicate treatments showed significantly higher Mn levels than the metalaxyl and control treatments.

Discussion

Metalaxyl provided greater protection for plants against chestnut ink disease, with no deaths recorded during the experimental period. Phosphorous acid (33% mortality)

and K-silicate (44% mortality) provided a higher degree of protection than the control treatment (78% mortality), but much lower than metalaxyl. None of the substances eliminated *Phytophthora* since the oomycete was found in the soil in all treatments at the end of the experiment. However, no symptoms of the disease were observed in the plants treated with metalaxyl. Metalaxyl has been considered one of the most effective substances in controlling *Phytophthora,* even in already infected plants (Akinsanmi & Drenth, 2013; Davidse, 1987; Erwin & Ribeiro, 1996; Schwinn et al., 1977), although its effectiveness depends on variables associated with the agrosystem (Guest & Bompeix, 1990).

The mortality of 33% of the plants in the phosphorous acid pots may have been the result of its application to the soil, which according to some authors may not have been the most effective method of protecting the plant roots. Phosphite (hydrogen phosphite, $H_2PO_3^-$), resulting from the deprotonation of phosphorous acid, is fully systemic in plants and can be translocated in both the xylem and the phloem (Guest & Grant, 1991;
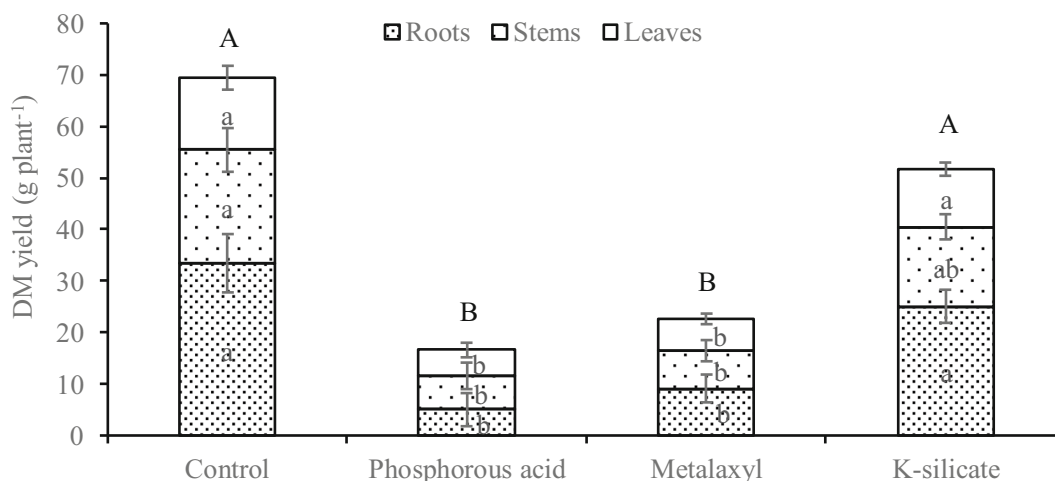

Fig. 1 Dry matter yield of surviving plants. To each plant part or total, means followed by the same letter (lowercase and uppercase, respectively) were not significantly different by Tukey HSD test ($\alpha = 0.05$); vertical bars are the standard errors

Table 2 *Phytophthora* detection in the different fungicidal treatments and trapping times

	24 h	48 h	72 h
Control	+	+	+
Phosphorous acid	−	+	+
Metalaxyl	−	+	+
K-silicate	−	+	+

+ *Phytophthora* detection in at least one disc of chestnut leaf; − no detection of *Phytophthora*

McDonald et al., 2001). Its great mobility in the plant allows it to be applied in different ways, including soil drenching, trunk injection or as a foliar spray (Akinsanmi & Drenth, 2013; Cohen & Coffey, 1986; Crane & Shearer, 2014; Masikane et al., 2020). Studies on apple trees performed by Nyoni et al. (2019) have shown that foliar applications of phosphite are more effective in providing phosphite to the roots than a soil drench. Darvas et al. (1984), for instance, reported that the most effective application method for phosphonates, in avocado, was trunk injection. Guest and Grant (1991) report that pathogen suppression occurs at higher phosphite concentrations (direct inhibition), while host plant resistance induction is associated with low phosphite concentrations (indirect inhibition). In addition, phosphorus acid or phosphite applied to the soil may lose efficiency in the long-term, since it can be converted to phosphate by soil microorganisms (du Jardin, 2012; McDonald et al., 2001). According to Gomez-Merino and Trejo-Telle (2015) this can occur within 3–

Table 3 Species identification and percentage of nucleotide identity of each identified species

Origen of detection	Species	Identity (%)
Control, pot 1	*Phytophthora cinnamomi*	99
Control, pot 2	*Phytophthora cinnamomi*	98
Control, pot 6	*Phytophthora cinnamomi*	99
Phosphorous acid, pot 2	*Phytophthora cinnamomi*	99
Phosphorous acid, pot 9	*Phytophthora cinnamomi*	100
Metalaxyl, pot 6	*Phytophthora cinnamomi*	100
Metalaxyl, pot 9	*Phytophthora cinnamomi*	99
K-silicate, pot 1	*Phytophthora cinnamomi*	99
K-silicate, pot 2	*Phytophthora cinnamomi*	99
K-silicate, pot 6	*Phytophthora cinnamomi*	99
K-silicate, pot 9	*Phytophthora cinnamomi*	99

4 months, a shorter period than the duration of this experiment.

The effect of Si in the control of plant diseases, its mode of action and its function in various pathosystems, is not yet fully understood. There is a hypothesis of a possible physical barrier formation, which is based on the accumulation of Si in plants, mainly in the cell wall. In its upward movement (apoplast movement), from roots to leaves, polymerization of Si occurs in the extracellular spaces, accumulating in the walls of the epidermal cells of the leaves and xylem vessels (Kim et al., 2002; Savvas & Ntatsi, 2015). Although in vitro studies carried out by Kaiser et al. (2005) reported 100% growth inhibition of *P. cinnamomi*, in this study the application to the soil of potassium silicate was not as effective, allowing a mortality of 44% at the end of the experiment.

Metalaxyl and, to a lesser extent, phosphorous acid treatments, protected the plants from death, but the surviving plants showed very poor growth performance compared to the surviving plants of the control treatment. Regarding metalaxyl, Sousa et al. (2017) reported a decrease in RuBisCO activity in *Solanun nigrum*, which resulted in photosynthesis inhibition and an abrupt decrease in plant growth. However, we think this was not the case in this experiment. A more consistent thesis will be provided with the discussion of the results of the concentration and recovery of nutrients in plant tissues and soil properties. Phosphite, in turn, can cause phytotoxicity in a wide range of species when present in plant tissues at high concentrations (Akinsanmi & Drenth, 2013; Barrett et al., 2004; Pilbeam et al., 2000). In addition, phosphite is not metabolized in cells, but recent investigations into biochemical and genetical mechanisms of phosphite action indicate that phosphite behaves as a phosphate analog, mimicking phosphate in plant cells but without making its P available for cell metabolism, inducing P deficiency (du Jardin, 2012). However, this second hypothesis does not seem to be a relevant cause for the reduction of dry matter yield in plants treated with phosphorous acid, considering the results of the tissue analysis that will be discussed later.

Ink disease of chestnut is endemic in Europe with cyclical resurgences, causing high tree mortality (Vannini & Vettraino, 2001). The disease has similar effects in Portugal. In the Trás-os-Montes, the biggest chestnut producing region of the country, 15% of the trees are affected by the disease (Gouveia, 2004). The detection technique (baiting trap) confirmed the presence

Table 4 Root nutrient concentration (average ± standard deviation) in the different treatments

Nutrient	Control	Phosphorous acid	Metalaxyl	K-silicate
Nitrogen (g kg^{-1})	6.1±1.08 c	22.9±1.32 a	14.4±0.65 b	5.8±0.36 c
Phosphorus (g kg^{-1})	0.8±0.05 b	3.9±0.33 a	1.1±0.15 b	1.0±0.13 b
Potassium (g kg^{-1})	3.5±0.28 b	5.1±0.52 b	5.1±0.20 b	7.3±1.14 a
Calcium (g kg^{-1})	2.5±0.33 a	2.5±0.45 a	3.0±0.21 a	2.6±0.13 a
Magnesium (g kg^{-1})	1.6±0.41 ab	2.1±0.17 a	2.1±0.07 a	1.4±0.12 b
Boron (mg kg^{-1})	19.6±3.37 b	29.6±0.34 a	25.5±2.31ab	23.2±2.41 b
Copper (mg kg^{-1})	9.4±1.62 a	11.3±1.26 a	10.8±1.68 a	11.1±1.68 a
Iron (mg kg^{-1})	2141.3±153.0 a	2195.4±205.8 a	1909.7±153.8 a	1875.4±100.9 a
Zinc (mg kg^{-1})	23.3±2.1 c	250.3±7.4 a	37.5±3.3 b	27.6±2.4 bc
Manganese (mg kg^{-1})	241.9±19.7 c	1233.9±73.9 a	706.6±66.0 b	261.4±18.4 c

Means followed by the same letter are not significantly different by Tukey HSD test (α = 0.05)

of *Phytophthora* in all soil samples analysed which included all treatments. This biodetection method has proven to be effective for the detection of *Phytophthora* from soil samples (Gouveia et al., 2009). The technique allowed the identification of *P. cinnamomi* as the dominant *Phytophthora* species present in the soil at the end of the experiment. In Portugal, three species of *Phytophthora* have been associated with the chestnut tree (*P. cinnamomi*, *P. cambivora* and the cryptic species *P. castanetorum*). However, *P. cinnamomi* is the species most commonly detected from soil samples and plant tissue from chestnut stands and it is considered the predominant pathogen in the development of ink disease (Fernandes, 1979; Gouveia, 2004), which is in agreement with the results of this study. Similarity of 98% with *P. cinnamomi* was obtained on root tissues from pot 2 (soil without any treatment) and also on the same pot at

the end of the experiment when identification of *Phytophthora* species was based on ITS sequences. However, percentages up to 99% similarity or even 100% were obtained in all other treatments or field control replications. Applying the "Species Hypotheses" based on interspecific similarities of between 97 and 99% (Hibbett et al., 2016) for Sequence Based Identification, all isolates were considered to be *P.cinnamomi* in the context of this work. Nevertheless, consistent differences in similarity among *Phytophthora* isolates justifies further studies to characterise *Phytophthora* species populations associated with chestnut ink disease in the Trás-os-Montes region. Morphological and physiological characteristics used in combination with DNA sequence data from the ITS, part of the nuclear heat shock protein 90 (HSP90), and β tubulin (Btub), mitochondrial cox1 and NADH genes can be considered, as used to characterize

Table 5 Leaf nutrient concentration (average ± standard deviation) in the different treatments

Nutrient	Control	Phosphorous acid	Metalaxyl	K-silicate
Nitrogen (g kg^{-1})	13.7±1.53 b	19.2±0.45 a	20.7±1.60 a	13.6±1.31 b
Phosphorus (g kg^{-1})	1.1±0.14 b	6.3±1.07 a	1.4±0.04 b	1.8±0.18 b
Potassium (g kg^{-1})	6.4±0.59 b	6.5±0.58 b	5.8±0.09 b	31.8±1.23 a
Calcium (g kg^{-1})	4.9±0.93 b	7.1±0.64 ab	8.2±1.65 a	2.0±0.06 c
Magnesium (g kg^{-1})	3.7±0.44 b	4.9±0.47 ab	6.1±0.83 a	1.4±0.40 c
Boron (mg kg^{-1})	64.8±12.20 b	186.1±12.43 a	179.4±19.80 a	72.6±7.40 b
Copper (mg kg^{-1})	8.1±0.96 ab	9.4±1.23 ab	10.5±1.07 a	7.8±0.52 b
Iron (mg kg^{-1})	232.2±23.13 a	84.7±7.59 b	92.4±5.78 b	103.5±12.04 b
Zinc (mg kg^{-1})	36.4±7.11 b	380.8±10.16 a	40.4±7.08 b	24.1±6.02 b
Manganese (mg kg^{-1})	1806.3±334.1 c	15,343.2±1883.5 a	8754.9±782.4 b	1115.8±310.3 c

Means followed by the same letter are not significantly different by Tukey HSD test (α = 0.05)

Table 6 Nutrient recovery in total plant dry biomass (average ± standard deviation) in the different treatments

Nutrient	Control	Phosphorous acid	Metalaxyl	K-silicate
Nitrogen (g kg^{-1})	258.4±33.34 a	241.6±22.76 a	318.7±29.65 a	271.5±19.04 a
Phosphorus (g kg^{-1})	27.5±2.43 ab	53.1±6.12 a	24.0±1.97 b	43.9±5.21 ab
Potassium (g kg^{-1})	146.0±12.82 b	61.4±7.93 b	100.5±9.95 b	550.3±62.34 a
Calcium (g kg^{-1})	113.1±9.78 a	60.5±7.09 a	105.0±11.46 a	88.9±7.90 a
Magnesium (g kg^{-1})	72.5±8.67 a	41.8±5.23 a	72.0±8.32 a	52.8±5.12 a
Boron (mg kg^{-1})	1.1±0.21 a	1.1±0.09 a	1.6±0.17 a	1.4±0.16 a
Copper (mg kg^{-1})	0.3±0.29 ab	0.1±0.01 b	0.2±0.01 ab	0.3±0.04 a
Iron (mg kg^{-1})	31.0±3.32 a	10.0±2.02 b	18.3±1.89 ab	36.2±2.98 a
Zinc (mg kg^{-1})	1.0±0.12 b	3.3±0.45 a	0.8±0.07 b	0.9±0.01 b
Manganese (mg kg^{-1})	26.4±3.45 b	82.8±10.04 a	69.4±8.41 a	19.4±2.54 b

Means followed by the same letter are not significantly different by Tukey HSD test ($\alpha = 0.05$)

new *Phytophthora* taxa present in Fagaceae forests in Austria, Italy and Portugal (Jung, Jung, Cacciola, et al., 2017b).

Tissue N concentration was noticeably higher in the phosphorous acid and metalaxyl treatments. However, plant N recovery was similar in all treatments. N is a limiting factor for plant growth, particularly in potted plants with reduced space for root expansion (Ferreira et al., 2020; Rodrigues et al., 2018), and can be taken up to luxury consumption levels when available in the soil

(Havlin et al., 2014). Thus, the lower N concentration in the control and K-silicate treatments was mainly the result of a dilution effect, which occurs due to the increase in plant biomass for the same amount of N available in the growing medium (Arrobas, Ferreira, et al., 2018b; Jarrel & Beverly, 1981).

The higher tissue P concentration in the phosphorous acid treatment in comparison to the other treatments was due to the P content in the commercial product. The result was corroborated by the increase in soil extractable

Table 7 Selected soil properties (average ± standard deviation) in the different treatments

Soil properties	Control	Phosphorous acid	Metalaxyl	K-silicate
[1]Organic C (g kg^{-1})	27.8±1.49 a	23.9±1.31 a	25.5±1.92 a	23.8±2.20 a
[2]pH$_{(H2O)}$	5.6±0.08 b	4.4±0.11 d	4.9±0.05 c	6.3±0.20 a
[3]Extrac. P (mg P$_2$O$_5$ kg^{-1})	105.7±5.74 c	174.1±6.66 a	107.2±3.15 c	135.8±11.80 b
[3]Extrac. K (mg K$_2$O kg^{-1})	308.3±17.56 b	304.7±24.19 b	314.0±21.07 b	1953.3±80.83 a
[4]Exch. Ca (cmol$_c$ kg^{-1})	2.8±0.14 b	2.0±0.11 c	2.6±0.18 b	3.2±0.15 a
[4]Exch. Mg (cmol$_c$ kg^{-1})	1.4±0.10 ab	0.9±0.18 c	1.1±0.13 bc	1.6±0.05 a
[4]Exch. K (cmol$_c$ kg^{-1})	0.6±0.23 b	0.6±0.15 b	0.6±0.05 b	3.8±0.04 a
[4]Exch. Na (cmol$_c$ kg^{-1})	0.6±0.04 a	0.4±0.06 c	0.5±0.02 bc	0.5±0.06 ab
[5]Exch. acidity (cmol$_c$ kg^{-1})	0.1±0.12 b	0.8±0.23 a	0.2±0.06 b	0.0±0.00 b
[6]CEC (cmol$_c$ kg^{-1})	5.6±0.35 b	4.7±0.18 c	5.0±0.32 bc	9.2±0.11 a
[7]Extrac. B (mg kg^{-1})	1.5±0.27 ab	1.2±0.10 b	1.4±0.31 b	2.1±0.13 a
[8]Extrac. Cu (mg kg^{-1})	2.2±0.60 a	1.7±0.09 a	1.7±0.39 a	1.9±0.03 a
[8]Extrac. Fe (mg kg^{-1})	108.3±7.24 b	121.3±19.61 ab	115.6±13.42 b	163.0±23.07 a
[8]Extrac. Zn (mg kg^{-1})	1.3±0.13 b	21.4±3.08 a	1.6±0.37 b	1.3±0.05 b
[8]Extrac. Mn (mg kg^{-1})	137.3±9.21 b	235.6±18.23 a	151.3±13.37 b	202.6±15.97 a

[1] Walkley-Black; [2] potentiometry; [3] ammonium lactate; [4] ammonium acetate, pH 7; [5] potassium chloride; [6] cation exchange capacity (exch. Bases + exch. acidity); [7] hot water, azomethine-H; [7] ammonium acetate and EDTA

Means followed by the same letter are not significantly different by Tukey HSD test ($\alpha = 0.05$)

P. After being added to the soil, the deprotonation of phosphorus acid makes phosphite available in the soil for plant uptake. Both phosphate and phosphite are acquired by plants via phosphate transporters (Jost et al., 2015). However, plants use phosphate and not phosphite in their metabolism. Oxidation of phosphite into phosphate, which could theoretically deliver usable P in the plant, is not documented in plant cells (du Jardin, 2012). However, in the soil, phosphite can undergo a gradual transformation, either by biological or non-biological oxidation. Soil microorganisms assimilate phosphite and release phosphate which can be taken up and used in plant nutrition. Phosphite oxidation to phosphate in soils lasts approximately 3–4 months (Gomez-Merino & Trejo-Telle, 2015), which is compatible with the use of P from phosphorus acid applications in plant metabolism. Thus, it is possible that the plants took up phosphite and phosphate during the growing period. This defeats the thesis that there may have been P deficiency in the plants. The high P values provided by tissue analysis are represented by all inorganic (phosphite and phosphate) and organic P forms in the plant. It is well-known that when high levels of soluble phosphate are present in the soil, plant roots can accumulate P in high amounts as a reserve (Ferreira et al., 2018; Rodrigues et al., 2021), which allows the buffering of P levels in the shoots. Thus, these particularly high levels of P in plant tissues, particularly in the roots, can be P in phosphite but also in metabolizable forms, which reduces the likelihood of phosphite toxicity.

The concentration of K in plant tissues, particularly in the leaves, was especially high in the K-silicate treatment. The increase of K in the tissues was a direct response to the presence of the element in the commercial product, also corroborated by the high K values found in the soil. The type of soil used in this experiment tends to provide enough K for plant growth (Arrobas, Afonso, & Rodrigues, 2018a; Arrobas et al., 2017) and it is unlikely that K per se played a relevant role in the performance of the plants or in their protection against Phytophthora.

Ca and Mg levels in plant tissues were also higher in the treatments producing less biomass. The result seems also to be a case of the dilution effect, although to a reduced extent in comparison with N, perhaps because their concentrations in plant tissues tend to vary less than usually occurs with N (Bryson et al., 2014).

Regarding micronutrients, there was observed a tendency for B to be at higher levels in the treatments with less biomass production. It should be noted that these types of soils usually provide insufficient amounts of B for the normal growth of plants (Arrobas et al., 2017; Rodrigues et al., 2020). The difference between treatments seems to be, as in the case of N, the result of a dilution effect. Regarding Fe, very high values were recorded in all treatments, especially in the roots. In pots, it is difficult to control water supply and aeration, which can reduce the redox potential in the soil, conditions which can lead to increased Fe bioavailability (Afonso et al., 2020). At excessive levels in the soil, Fe appeared concentrated in the roots as reported by Rodrigues et al. (2021). The levels of Zn were significantly higher in the phosphorous acid treatment, which were probably the result of the presence of the element in the commercial product. The levels of Mn were very high in the leaves and roots in all treatments. It is well-known that chestnut accumulates high amounts of Mn in its tissues when grown in acidic soils where the element is found at high levels (Arrobas, Afonso, & Rodrigues, 2018a; Arrobas et al., 2017). However, the values were particularly high in the phosphorus acid treatment due to the great reduction in soil pH caused (the original pH of the product is 0.3), which solubilized large quantities of Mn. The commercial product also contains Mn, which also might have contributed to the increase of Mn in plant tissues. The metalaxyl treatment also caused a strong decrease in soil pH, although less than that caused by phosphorous acid, and the Mn levels were much higher than in the control, but lower than in the phosphorus acid treatment. Thus, the marked decrease in plant growth in the phosphorous acid treatment followed by the metalaxyl treatment was probably due to Mn toxicity caused by the strong acidification of the soil. It should be noted that the higher limit of the sufficiency range for leaf Mn concentration is 2000 mg kg^{-1} (Arrobas, Afonso, & Rodrigues, 2018a). In this study, the values exceeded 15,000 mg kg^{-1} in the phosphorous acid, and 8000 mg kg^{-1} in the metalaxyl, treatment.

Conclusions

The metalaxyl treatment provided great protection of plants against Phytophthora with no deaths recorded. However, under the conditions of this experiment, it resulted in a significant decrease in the plant growth performance, most likely due to the strong acidification of the soil which resulted in Mn toxicity. Phosphorous

acid also greatly reduced plant death compared to the control, but plant growth was even more depressed than in the metalaxyl treatment as a result of a further lowering of the pH which caused an even more severe toxicity of Mn. Despite the effectiveness shown by these substances in reducing plant death caused by *Phytophthora*, it is also important to look at the concentration of the solutions, or the forms of the active ingredients, to reduce the risk of excessive soil acidification. This is particularly important for management strategies when foci of *Phytophthora* root rot suddenly appear, and it is necessary to limit the damage and prevent the natural spread of the inoculum. In the case of phosphorous acid, perhaps the application of their salts (phosphites) is a way of maintaining the positive results of plant protection while avoiding the problem of soil acidification. The use of K-silicate did not show such negative effects on the growth of the treated plants but gave less protection against *Phytophthora* in comparison to phosphorous acid and especially to metalaxyl. *Phytophthora* root rot management is most successful when active ingredients with different modes of action are integrated with a set of preventive and cultural practices over the long lifespan of chestnut trees.

Compliance with ethical standards

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent Informed consent was not applicable to this article since no information regarding individual participants was included in the study.

Conflict of interest The authors declare no conflict of interest.

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