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Calcium chelate is as effective as phosphite in controlling Phytophthora root rot in glasshouse trials

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Abstract

Species in the genus *Phytophthora* cause significant economic losses in crops and damage to forests and natural ecosystems worldwide. Currently, phosphite is the most effective chemical for disease management, but excessive phosphite concentrations can result in phytotoxicity in plants and the development of tolerance by the pathogen. Two newly developed metal chelates and phosphite (alone and in combination) were tested for their in vitro and in planta efficacy against *Phytophthora cinnamomi*. In glasshouse trials, 0.25% and 0.5% of each chemical treatment (phosphite, Ca chelate, Zn chelate) and Ca chelate + phosphite were used as a foliar application on 3-month-old seedlings of *Banksia grandis* (experiment not repeated) and *Eucalyptus marginata*, prior to inoculation with *P. cinnamomi*. All noninoculated control plants remained healthy, while significant root damage and reduction of dry root weights were observed for inoculated untreated plants. Individually, phosphite and Ca chelate significantly reduced root lesion development of *P. cinnamomi* compared to the control, with Ca chelate attaining superior results to phosphite at the same concentration. In combination, Ca chelate + phosphite had the largest reduction in root lesion development in both plant species; however, this result has not yet been replicated but did reflect previous in vitro results. The Zn chelate applications were not effective. Ca chelate has the potential to be developed as a fungicide to control *Phytophthora* species.

KEYWORDS
calcium, chemical control, disease suppression, induced resistance, phosphite

1 | INTRODUCTION

Phytophthora is a genus of plant-damaging oomycetes, which cause significant economic losses and environmental damage. Worldwide, *Phytophthora cinnamomi* is one of the most invasive plant pathogens, causing severe disease and mortality to ornamental and native plants in agriculture, horticulture and forestry (Burgess et al., 2021). Of the management options used to reduce the impact and severity of diseases, cultural and chemical control are the most successful. Cultural control includes managing drainage, sanitation and clean plant stock (Strömberg & Brishammar, 1991). Chemical control is most commonly used due to its rapid action, high efficiency and cost-effectiveness (Gisi, 2002). Formulations containing phosphite are very effective systemic fungicides used to control many

plant diseases caused by *Phytophthora* species (Hardy et al., 2001; Gómez-Merino & Trejo-Téllez, 2015). Phosphite is the anionic form of phosphonic acid (HPO_3^{2-}) and has the most success when applied as a foliar spray or by trunk injection (Guest & Grant, 1991; Hardy et al., 2001). It acts directly on the pathogen and indirectly through host defence stimulation (Jackson et al., 2000). However, excessive phosphite can result in phytotoxicity in horticultural crops (Seymour et al., 1994) and native species (Tynan et al., 2001). Some *Phytophthora* species tolerate phosphite (Barrett et al., 2004; Veena et al., 2010). For example, isolates of *P. cinnamomi* are developing tolerance to phosphite in avocado orchards that routinely use phosphite (Dobrowolski et al., 2008). Other fungicides such as metalaxyl, mefenoxam, dimethomorph and cymoxanil have been used against oomycete pathogens; however, *Phytophthora* species have developed resistance to these fungicides (Parra & Ristaino, 2001; Thomidis & Elena, 2001). Unlike phosphite, most fungicides do not stimulate plant defence against *Phytophthora* species, so their effect does not persist, requiring frequent applications. Many are also toxic to humans. The European Commission's Farm to Fork Strategy is pushing agriculture to reduce pesticide use by 50% before 2050. This means there is a need to develop new benign fungicides to ensure ongoing protection of plants from disease and reduce the risk of exposure to toxic chemicals in humans.

Bio-inorganic chemical research has highlighted the potential role of metal complexes as chemical treatments against microorganisms. Metal complexes or metal chelates are structures composed of a metal atom in the centre that bonds with the molecules or ions surrounding it. Complexes of transition metals (from groups 3–12 of the periodic table) that have more than one oxidation state and nontransition metals are now widely used in therapeutic fields and have been used as antimicrobial treatments (Chai et al., 2017; Shebl et al., 2017; Taghizadeh et al., 2017) and anticancer agents (van Rijt & Sadler, 2009). Concerning plant pathogens, few studies have used metal complexes to control pathogens and the diseases they cause (Liu et al., 2018; Smaili et al., 2017).

Calcium has several impacts not only on *Phytophthora* species but also on plants. Calcium can suppress release of *Phytophthora* zoospores and reduce zoospore motility (von Broembsen & Deacon, 1997). Calcium can boost plant resistance to various pathogens, including *Phytophthora* (Kao, 1986; Lin et al., 1990). For instance, root damage caused by *P. cinnamomi* has been reduced by a soil amendment of 30 mM calcium sulphate combined with a foliar treatment of 0.3% phosphite, providing 100% survival of *Banksia leptophylla* 12 months after inoculation with *P. cinnamomi* compared to 3% survival of the untreated control (Stasikowski et al., 2014). These authors proposed a model involving the inhibition of calcium-dependent ATPases by phosphite and pyrophosphate, and the subsequent disruption of calcium ion homeostasis and signalling in the cell, resulting in the stimulation of plant defences. This indicates promising effects resulting in positive feedback mechanisms that will benefit plant survival. Unfortunately, calcium (and calcium salts) are immobile in plants. However, calcium can be mobilized in plants by the chemical ligands in chelate.

Zinc, a transition metal, plays a crucial role in growth, development and defence (Cabot et al., 2019; Dordas, 2008). Cabot et al. (2019) list key functions of plant defence that are regulated by zinc, including alcohol dehydrogenase and carbonic anhydrase (which is involved with the salicylic acid-binding protein), metallothionein, superoxide-dismutase (SOD) and Zn finger. Zn also acts directly on pathogen virulence, for example, as an effector of α-mannosidase (Martínez-Cruz et al., 2018). Therefore, zinc is hypothesized to be a potential stimulator of plant defences against *Phytophthora*.

As both Ca and Zn have the potential to help protect plants from pathogens, calcium and zinc mixed ligand complexes have been produced and tested in vitro for their efficacy against *P. cinnamomi* (Mahmood, 2021). The current study aimed to evaluate, in planta, the efficacy of these complexes in controlling *Phytophthora* species compared to and in combtion with phosphite.

2 | MATERIALS AND METHODS

Three glasshouse trials were undertaken. The first trial examined the effect of treatments (Ca chelate, Zn chelate and phosphite) on *Eucalyptus marginata*; the second trial, also with *E. marginata*, included a combined treatment with phosphite and the calcium chelate; the third trial with *Banksia grandis* omitted the Zn chelate treatments. The second and third trials were run concurrently. These plant species are native to the southwest of Western Australia (WA) and are susceptible to *P. cinnamomi* (McDougall et al., 2001; Tynan et al., 2001). Seeds were obtained from Nindethana Seed Service P/L and germinated in trays in the glasshouse. Germinants were transferred to individual 1.5 L pots (Garden City Plastics) containing washed river sand, steam pasteurized for 2 h at 65°C. The pots had a sterile 10 ml plastic tube pressed into the sand before planting to retain the space for later insertion of the *Phytophthora* inoculum, minimizing any root damage.

The inoculum of *P. cinnamomi* isolate MP94-48 was prepared using a previously described vermiculite/millet seed-based inoculum (Belhaj et al., 2018). The inoculum was incubated at 22°C for 4 weeks in the dark and shaken weekly to distribute the pathogen evenly through the substrate. Soil inoculation of the plants occurred 48 h after the foliage was sprayed with chemical treatments. The plastic tube was removed, and 15 g inoculum (1% of the weight of sand in the pot) was inserted. Half of each spray treatment (seven pots) was inoculated with *P. cinnamomi* inoculum, while sterile vermiculite was used for the noninoculated controls. The pots were flooded for 12 h so that water reached the soil's surface. The flooding was repeated every 2 weeks, with the pots free-draining between flooding events. Pots were arranged in a randomized complete block design in an evaporatively cooled glasshouse at Murdoch University at a temperature between 15.6 and 27.2°C. Plants were watered daily with deionized water and fertilized weekly with 2 g of Thrive (Yates)/5 L water.

Ca and Zn chelates were prepared as described by Mahmood (2021). For the first trial, 3 months after the seed of

E. marginata was sown, seedlings were sprayed with one of nine treatments: 0.1%, 0.25% or 0.5% Ca chelate, Zn chelate and phosphite combined with the adjuvant BS1000 (Crop Care Australasia Pty Ltd) at 130 µl/L. In the late afternoon, pots of each treatment were sprayed with 300 ml of chemical solution using a small spray bottle. The plants were sprayed to run-off, and the container substrate was covered with plastic to prevent the chemicals from reaching the sand. There were 10 replicates per chemical treatment, and 10 control plants were sprayed only with water. Two days later, half of the plants were inoculated with *P. cinnamomi*.

Eight weeks after inoculation, the plants were harvested following the protocol of Migliorini et al. (2019) and *P. cinnamomi* was reisolated from infected plants. The shoots and roots were separated, and care was taken to wash the sand from the roots, which were then scored for root damage. The root damage was rated as 0 = no damage to 4 = severe root damage. Damage was considered to be any discolouration or necrotic tissue on the roots. The roots and shoots were dried at 37°C for 7 days and then at 60°C for 24 h. Representative samples were weighed on consecutive days, and when no difference in weight was obtained, all the dry weights were recorded. *P. cinnamomi* was recovered from the fresh roots of inoculated plants by plating them onto NARH, a *Phytophthora* selective medium (Sarker et al., 2020). The trial was conducted between February and July 2017, with mean maximum and minimum temperatures of 26.6 and 15.6°C, respectively.

For the second and third trials, 3 months after the seeds of *B. grandis* and *E. marginata* were planted, seedlings were sprayed with one of six treatments: 0.25% and 0.5% phosphite, 0.25% and 0.5% Ca chelate and two combinations, 0.125% Ca chelate + 0.125% phosphite and 0.25% Ca chelate + 0.25% phosphite. There were two additional treatments for *E. marginata*: 0.25% and 0.5% Zn chelate. Fourteen replicates per chemical treatment and 14 control plants of each species were sprayed only with water. Two days later, half the plants were inoculated with *P. cinnamomi*. These trials were conducted and harvested using the same method as the first experiment. The trials were conducted between August 2017 and February 2018, with mean maximum and minimum temperatures of 27.2 and 15.8°C, respectively.

The glasshouse trials were analysed separately. Analysis was performed in R (R Core Team, 2021). Before analysis, the data were explored for violation of assumptions (normality, heterogeneity, independence, sphericity, interactions). Where assumptions of normality were violated (for the root ratings), Kruskal–Wallis and Dunn tests were used. Where assumptions were satisfied (root and total dry weight), data were analysed by two-way analysis of variance (ANOVA) with post hoc Tukey test performed for pairwise comparisons.

3 | RESULTS

In the first trial with *E. marginata*, no root damage was observed for the noninoculated control treatments. The lowest root damage

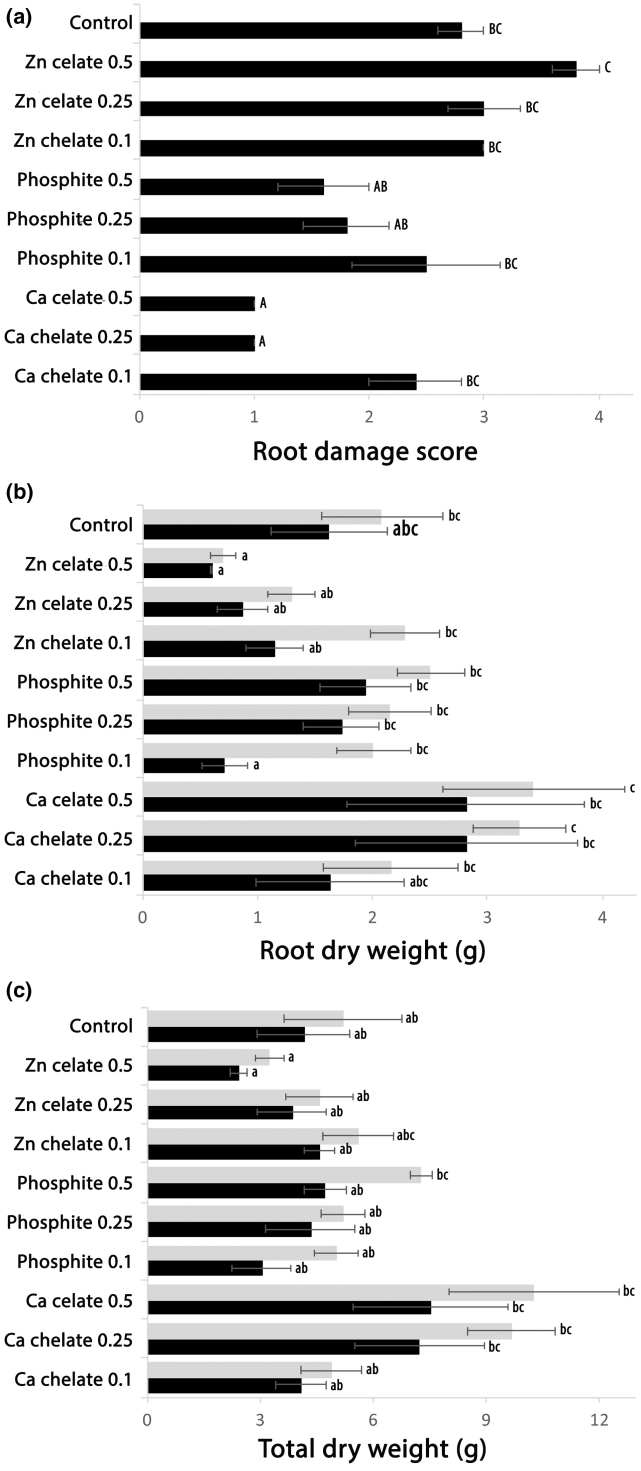

FIGURE 1 The effect of foliar treatments on disease development caused by *Phytophthora cinnamomi* in *Eucalyptus marginata*. (a) Root damage score, (b) root and (c) total dry weights of inoculated (dark grey) and noninoculated (light grey) seedlings (±*SE*; $n = 5$). For the root damage score, 0 = no damage to 4 = severe damage; all noninoculated controls were rated as 0 as no damage was observed. The total concentration is shown for the Ca chelate + phosphite mix, with equal amounts of each. Within each graph, bars with the same letter are not significantly ($p \leq 0.05$) different. All concentrations are percentages.

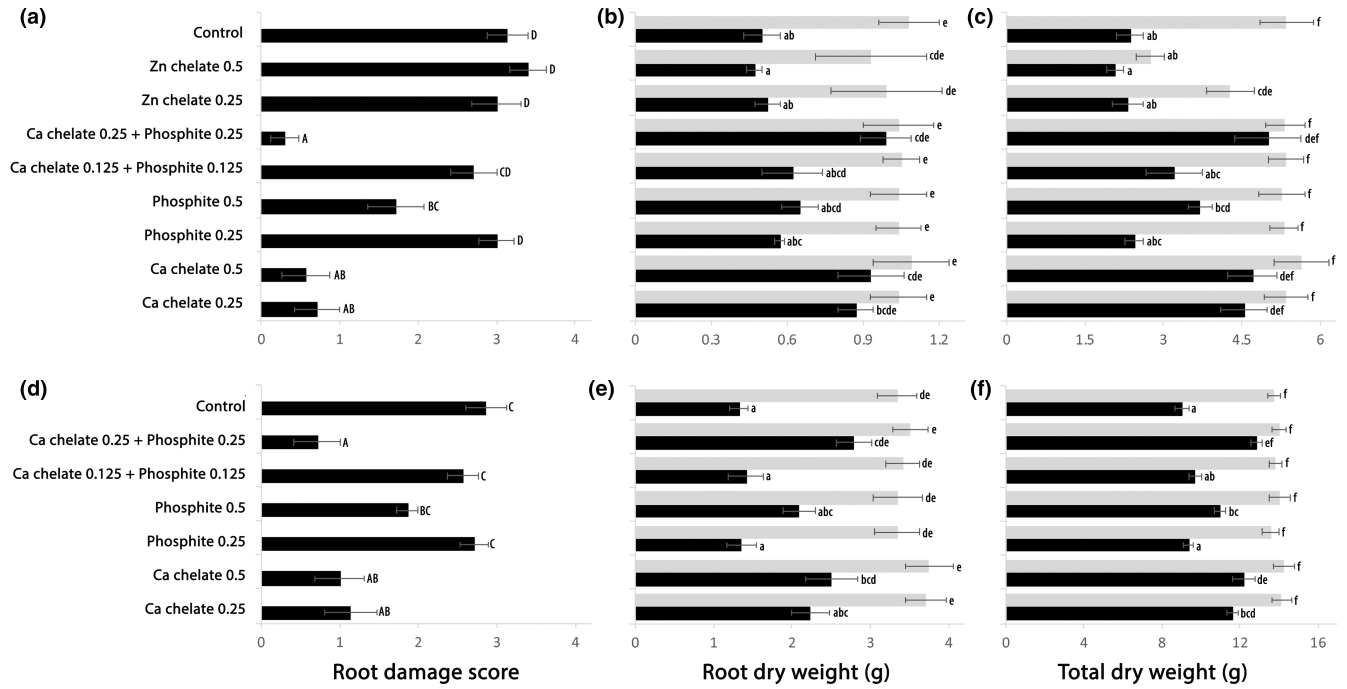

FIGURE 2 The effect of foliar treatments on disease development caused by *Phytophthora cinnamomi* in (a–c) *Eucalyptus marginata* and (d–f) *Banksia grandis*. (a, d) Root damage score, (b, e) root and (c, f) total dry weights of inoculated (dark grey) and noninoculated (light grey) seedlings (±*SE*; $n = 7$). For the root damage score, 0 = no damage to 4 = severe damage; all noninoculated controls were rated as 0 as no damage was observed. Within each graph, bars with the same letter are not significantly ($p \leq 0.05$) different. All concentrations are percentages.

scores observed for plants inoculated with *P. cinnamomi* were for those treated with Ca chelate at 0.25% and 0.5%, followed by phosphite at 0.25% and 0.5% (Figure 1a). Treatments at 0.1% and all Zn treatments were not significantly different from the control ($p > 0.05$; Figure 1a). There were significant differences between treatments in root weight ($p = 0.001$) and total plant dry weight ($p < 0.001$). For noninoculated plants, the zinc treatments at 0.25% and 0.5% resulted in significantly reduced root growth compared to the control plants (Figure 1b). For inoculated plants, the root biomass of the calcium treatments at 0.25% and 0.5% were larger than for the control inoculated plants, although the variability was high, and thus the results were not significant. For noninoculated and inoculated plants, total dry weight was greater for plants treated with 0.25% and 0.5% calcium chelate (Figure 1c), although again this was not significant due to large standard errors.

The second trial's results were very similar (but more significant due to the increased number of biological replicates) to those in the first trial for the independent application of the two metal chelates and phosphite treatments; observations for *E. marginata* were confirmed and paralleled by those for *B. grandis* in the third trial. Ca chelate alone and in combination with phosphite (0.25% Ca chelate + 0.25% phosphite) significantly ($p < 0.05$) reduced root damage and gave the highest root and plant dry weights for both *E. marginata* and *B. grandis* (Figures 2a,d, 3 and 4). For *E. marginata* the 0.5% phosphite treatment also significantly ($p < 0.05$) reduced root damage (Figure 2a).

Inoculation with *P. cinnamomi* significantly ($p < 0.005$) reduced the dry root weights for most treatments for both plant species (Figure 2b,e). However, there was no significant difference (i.e., the treatment protected the roots from infection) for both species with the higher concentration of the combination treatment; 0.25% Ca chelate +0.25% phosphite. Additionally, both Ca chelate treatments protected the roots of *E. marginata* (Figure 2b). The same results were observed for the total dry weight (Figure 2c,f).

All untreated noninoculated (positive control) plants of *B. grandis* (Figure 2e) and *E. marginata* (Figure 3e) remained healthy for the duration of the trial, and no *P. cinnamomi* was isolated from them. *P. cinnamomi* was isolated from the roots of all inoculated plants and from the 10 inoculated plants that died during the experiment: two untreated *E. marginata*, three untreated *B. grandis* and five plants of *E. marginata* treated with Zn chelate.

4 | DISCUSSION

P. cinnamomi caused disease in the untreated controls in planta within 8 weeks, and this root damage was reduced significantly by all treatments except for Zn chelate. The Ca chelate was more effective than phosphite in reducing the damage of *P. cinnamomi* to plants. However, when Ca chelate was applied with phosphite, there was a potential synergistic effect with a greater reduction in the impact of *P. cinnamomi* than when either treatment was applied alone. More

FIGURE 3 The effect of foliar chemical treatments applied on the impact of *Phytophthora cinnamomi* on roots of *Eucalyptus marginata*. The controls are untreated; the negative control is inoculated (a), while the positive control is not inoculated (e). Plants inoculated with *P. cinnamomi* were treated with phosphite (b, f), calcium chelate (c, g), or phosphite + calcium chelate (d, h). All concentrations are percentages.

work must be done to investigate this under glasshouse and field conditions.

The phosphite treatment results agreed with previous studies where phosphite reduces root rot, with the documented modes of action through both the stimulation of plant defence responses and/or inhibition of pathogen growth (Fenn & Coffey, 1984; Guest & Grant, 1991; Jackson et al., 2000). Contrary to expectations, Zn chelate did not control *P. cinnamomi* in vitro (Mahmood, 2021) nor in planta. Instead, it caused phytotoxicity in the noninoculated control plants of *E. marginata* resulting in greater root damage and lower root weights than the inoculated untreated control plants. At high concentrations, Zn can detrimentally impact plant growth, photosynthetic activity, water relations and metabolism (Broadley et al., 2007).

The present study shows that 0.5% phosphite reduced root damage by *P. cinnamomi*, but not to the same degree as the Ca chelate at 0.25% or 0.5%, or when 0.25% phosphite and 0.25% Ca chelate were combined. The calcium ions of the chelate might stimulate plant defences (Stäb & Ebel, 1987; Sugimoto et al., 2008) and may inhibit the growth of *Phytophthora* species by suppression of

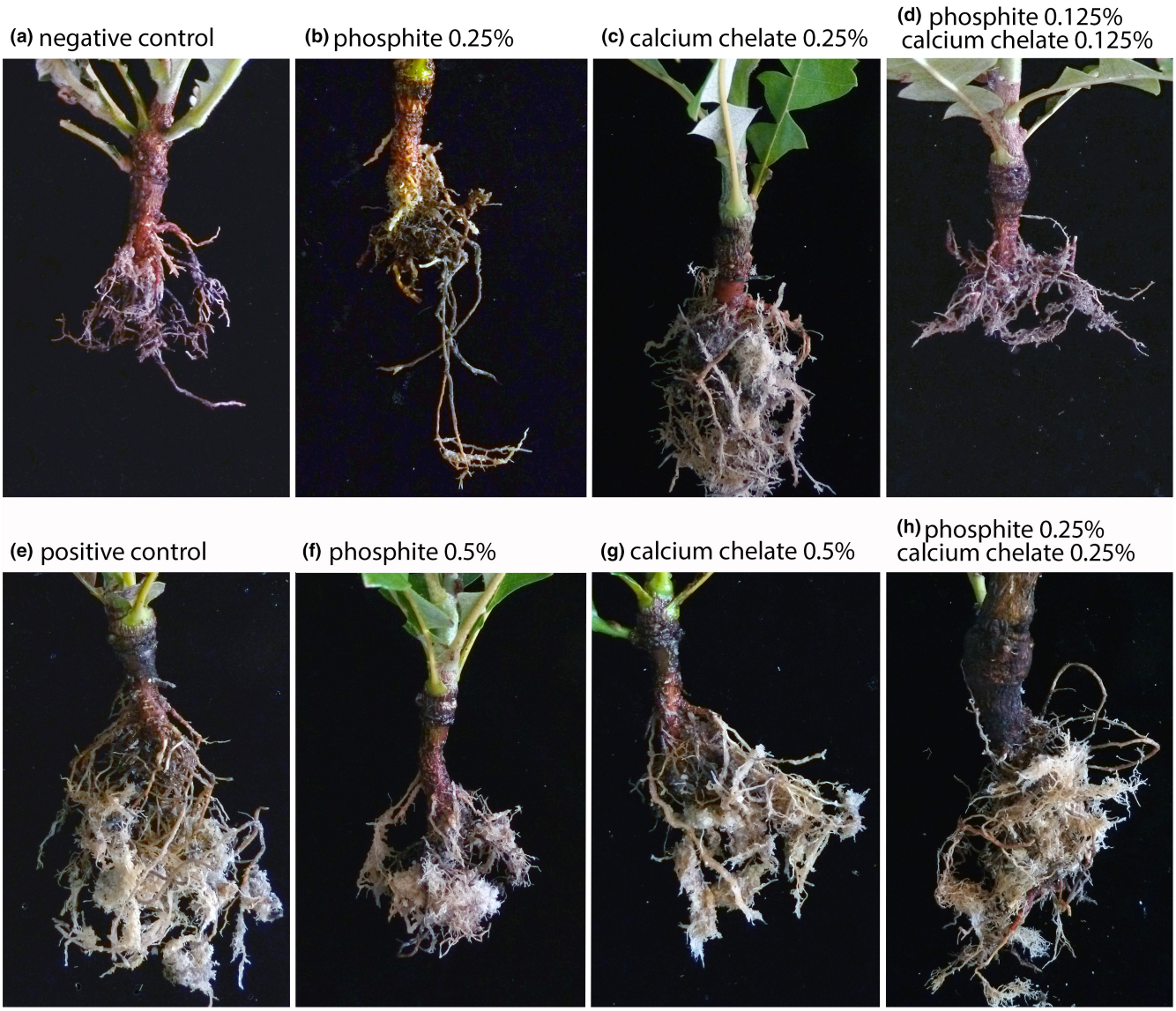

FIGURE 4 The effect of foliar chemical treatments applied on the impact of *Phytophthora cinnamomi* on roots of *Banksia grandis*. The controls are untreated; the negative control is inoculated (a), while the positive control is not inoculated (e). Plants inoculated with *P. cinnamomi* were treated with phosphite (b, f), calcium chelate (c, g), or phosphite + calcium chelate (d, h). All concentrations are percentages.

sporangia formation (Messenger et al., 2000; Serrano et al., 2012), or a combination of both (Sugimoto et al., 2008). For both plant species trialled, spraying with a combined treatment of 0.25% Ca chelate + 0.25% phosphite resulted in the least root rot. This treatment combination also had the greatest impact on *P. cinnamomi* in vitro (Mahmood, 2021), indicating a potential synergistic effect of Ca chelate and phosphite. In pot trials, Stasikowski et al. (2014) reported only 2.7% survival of *B. leptophylla* (without treatment), compared to 53% survival with 0.3% phosphite treatment and 100% survival when treated with 0.3% phosphite (foliar) and 30 mM calcium (soil addition). Both phosphite and calcium ions produced positive feedback mechanisms, enhancing the action of each treatment on plant defence, reducing the pathogen's ability to infect, reproduce and survive within plants. They explain that phosphite inhibits the ATPase reaction (dependent upon Ca^{2+}), which stops removing the ion from the cytosol, increasing the concentration and thus potentially eliciting a signal for the coordinated response of the plant. The phosphite and Ca chelate combinations may have worked directly and indirectly on the pathogen in the current study. However, studies examining plant defences, such as the up- or down-regulation of known defence genes (Burra et al., 2014; Kasuga et al., 2021), would need to be conducted to prove this hypothesis.

This study provides strong evidence for the potential of the formulated Ca chelate to be used alone or in combination with phosphite to enhance the current control of *P. cinnamomi* from phosphite alone. It is now vital to consider the longevity of the effect of these treatments. The concentration in planta should be tested in the roots and leaves of plants of different ages. Also, a combination of 0.5% Ca chelate and 0.5% phosphite should be

tested to determine if the control of *P. cinnamomi* in planta is further improved. It will be important to investigate the efficacy of the Ca chelate towards other *Phytophthora* species and its efficacy and persistence in a broad range of plant species, particularly native plants. It is imperative to take the findings from these studies into the field. Further tests should ascertain if Ca chelate affects the metabolism of *Phytophthora* species and determine the mode of action of the Ca chelate in plants. Ca chelate shows promise as another product that we can utilize to stimulate plant defence responses against plant pathogens.

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DATA AVAILABILITY STATEMENT

The data supporting this study's findings are available from the corresponding author upon reasonable request.

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