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Evaluating the potential of environmentally friendly compounds to deactivate different life stages of *Phytophthora* species

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Abstract

Phytophthora species are a genus in the oomycete class and are destructive plant pathogens. The ability to inhibit all stages in the life cycle of these aggressive *Phytophthora* pathogens is essential for the long-term protection of native trees, plantation forests and horticultural crops that they threaten. Compounds having this ability are currently scarce. In this study, antifungal compounds of natural and synthetic origin were screened against the mycelial, zoospore and oospore life stages of *Phytophthora agathidicida* (*P. agathidicida* 18406), *P. multivora* (*P. multivora* 20280) and *P. cinnamomi* (*P. cinnamomi* 20276, 3910 and 21050). An antifungal lipopeptide showed promising (25–200 μg/mL) antimycelial activity against all the tested *Phytophthora* species, rapid (c. 30 s) inhibition of *P. agathidicida*, *P. multivora* and *P. cinnamomi* 3910 zoospore motility, inhibition of zoospore germination at 12.5–50 μg/mL and reduced the viability of *P. agathidicida* and *P. multivora* oospores at 1000 μg/mL. Polygodial extracted from New Zealand horopito leaves showed more potent (5–50 μg/mL) antimycelial activity and inhibited zoospore motility of *P. agathidicida*, *P. multivora* and *P. cinnamomi* 3910, although at a slightly slower rate; however, it was inactive against oospores. Polygodial and lipopeptide protected kauri and avocado leaves from *Phytophthora* infection and did not show symptoms of phytotoxicity. Polygodial also inhibited the growth of *P. agathidicida* in soil. This study demonstrates for the first time the promising antioomycete potential of compounds ranging from small molecule natural products to larger biomolecules such as lipopeptides, which can be developed as compounds to deactivate the different life stages of aggressive *Phytophthora* species.

KEYWORDS
antifungal lipopeptides, antimycelial, antioospore, antizoospore, *Phytophthora*, polygodial

1 | INTRODUCTION

Phytophthora species belonging to the oomycete (fungus-like organisms) class are one of the most devastating plant pathogens responsible for causing damage to natural ecosystems (e.g., oak, eucalyptus, kauri) and economically relevant crops (potato, tomato, pepper, avocado and pineapple) globally. Currently, more than 200 *Phytophthora* species have been formally identified and many of these are considered to be pathogenic (Abad et al., 2022). *Phytophthora* species infect different parts of the plant such as roots, trunks and foliage causing a range of symptoms such as chlorosis, dieback, root rot and rotting of other organs. Some *Phytophthora* species such as *P. cinnamomi* have a broad host range and can infect approximately 5000 plant species including crops such as pineapple and avocado and native flora such as eucalyptus and oak (Hardham & Blackman, 2018). By contrast, species such as *P. agathidicida* may have a narrow host range by mainly infecting kauri (*Agathis australis*) (Bradshaw et al., 2020), an endemic tree to New Zealand (Steward & Beveridge, 2010).

Management of *Phytophthora* species is notoriously difficult due to the multiple life stages of the pathogen, particularly the mycelium (vegetative growth stage), zoospores (responsible for inoculating new hosts) and the long-living oospores embedded in soil that are responsible for the persistence of the pathogen in the environment for several decades (Judelson & Blanco, 2005). The current disease management strategies to contain *Phytophthora* infections involve horticultural practices such as cutting or burning of infected host plants and creating buffer zones around infected areas and the use of several chemical pesticides such as copper, phosphite, metalaxyl, fosetyl-Al, tertiary amines like sterigene and the recently approved new class of fungicide, oxathiapiprolin (Bellgard et al., 2010; Garbelotto et al., 2009; Hardy et al., 2001; Miao et al., 2020; Smillie et al., 1989). However, some of these commonly used compounds cause phytotoxicity at higher concentrations (Horner et al., 2015). Continuous use also leads to *Phytophthora* developing resistance to the treatments (Dobrowolski et al., 2008; Hunter, Williams, et al., 2018). Additionally, they are not biodegradable and none of these compounds except oxathiapiprolin (Lacey et al., 2021) are reported to be active against multiple life stages of *Phytophthora* (Dick & Kimberley, 2013). Resistance to oxathiapiprolin by some *Phytophthora* has been reported since its approval as a fungicide in 2016 (Miao et al., 2020). Therefore, novel, nonphytotoxic disease control options with activity against multiple life stages of *Phytophthora* without the possibility of developing resistance over time are urgently needed.

Oomycetes share similarities with fungi such as the formation of mycelia and the ability to sporulate. Both also have thick cell walls, although the fungal cell wall is mainly composed of chitin whilst the oomycete cell wall contains cellulose. Unlike fungi, which heavily rely on sterol biosynthesis as it forms the major component of the fungal cell membrane, *Phytophthora* does not require sterols. Although some antifungal agents that target chitin biosynthesis (polyoxin) or sterol synthesis (propiconazole) are not effective against *Phytophthora* species, fungicides with membrane lytic mechanisms have been well documented in the literature to exhibit anti-*Phytophthora* activity (Kim et al., 2000; Rajasekaran et al., 2001; Tran et al., 2007). Thus, it seemed logical to explore the antioomycete potential of known antifungal compounds, especially with membrane lytic mechanisms, in an effort to further develop them to combat *Phytophthora*.

Antifungal peptides offer the advantages of broad-spectrum antimicrobial activity and biodegradability. They are less prone to resistance development because of their membrane lytic mechanisms (De Lucca & Walsh, 1999). Although antifungal peptides with anti-*Phytophthora* activity have been reported in the literature, none of these have been shown to be effective against the different life stages of *Phytophthora* (Rajasekaran et al., 2001; Tran et al., 2007). We have previously reported on novel antifungal lipopeptides (De Zoysa et al., 2018) with activity against the fungal pathogen *Candida albicans*. These lipopeptides were active against the planktonic and preformed mycelial biofilms of *C. albicans* while maintaining negligible haemolysis and cytotoxicity to human cells (De Zoysa et al., 2018). These lipopeptides lyse fungal membranes within a few minutes, which make them ideal candidates to target the zoospores of *Phytophthora*, which are generally short-lived.

Natural products form a rich source of bioactive compounds and have shown promising activity against a range of *Phytophthora* species (Lawrence et al., 2019; Mondol et al., 2015; Strobel et al., 1999). Horopito (*Pseudowintera*) is an endemic shrub of New Zealand with at least four known species: *P. colorata* (mountain horopito or pepper tree), *P. axillaris* (lowland horopito), *P. traversii* (less common) and *P. insperata* (threatened species) (Wayman et al., 2010). Sesquiterpene dialdehyde compounds isolated from horopito, particularly from *P. colorata* and *P. axillaris*, have a range of biological activities such as antibacterial, antifungal, antifeedant and antihyperalgesic properties (Wayman et al., 2010). Polygodial is the most frequently identified sesquiterpene dialdehyde from horopito with noticeable biological activity (McCallion et al., 1982; Wayman et al., 2010). Synthetic derivatives of polygodial with known antifungal (Derita et al., 2013) and antifeedant (Manwill et al., 2020) activities have been reported in the literature. In this paper, we investigate the anti-*Phytophthora* activity of antifungal lipopeptides, polygodial and two synthetic derivatives (diol and dicarboxylic acid) of polygodial (Figure S1) against multiple life stages (mycelia, zoospores and oospores) of *Phytophthora* species. *P. agathidicida* is the main pathogen responsible for causing kauri dieback (Bradshaw et al., 2020). *P. cinnamomi* (*P. cinnamomi* 20276, *P. cinnamomi* 3910) and *P. multivora* (*P. multivora* 20280) have also been isolated from kauri roots, although these species are considered less aggressive than *P. agathidicida*. Kauri holds great ecological and cultural significance to New Zealand. *P. cinnamomi* 21050 has been isolated from avocado and is known to cause *Phytophthora* root rot, considered the most common and deadly disease affecting the global avocado industry.

2 | MATERIALS AND METHODS

2.1 | *Phytophthora* isolates and cultivation

Phytophthora agathidicida isolate ICMP 18406 (kauri), *P. cinnamomi* isolates ICMP 20276 (kauri) and ICMP 21050 (avocado) and *P. multivora* isolate ICMP 20280 (kauri) were purchased from Manaaki

Whenua-Landcare Research (New Zealand). *P. cinnamomi* isolate NZFS 3910 (kauri) was provided by Scion (New Zealand). *Phytophthora* isolates were cultured for assays by growing a single mycelial agar plug (3 mm diameter) at 24°C on clarified 20% V8 agar plates for 7 days. Pond water and garden soil for the zoospore experiment were collected from Auckland Domain and a residential area, respectively.

2.2 | Compounds screened

The antifungal lipopeptides (Figure S1; Compounds 1–3) were synthesized following standard Fmoc/*t*Bu solid phase peptide synthesis protocols as reported in detail in our previous publication (De Zoysa et al., 2018). Agri-fos 600 (phosphite source) was purchased from Key Industries. Synthetic polygodial was purchased from Sigma-Aldrich. Polymyxin B, a clinically used lipopeptide antibiotic against multi-drug resistant gram-negative bacteria, and chlortetracycline antibiotics were purchased from AK Scientific. Crushed horopito (*P. colorata*) leaves were obtained from Forest Herbs Research Ltd.

2.2.1 | Polygodial extraction (Just et al., 2015)

Horopito leaves (105 g) were dried overnight at 37°C. The dried leaves were finely ground using a coffee grinder (Breville). The extraction followed the protocol reported in the literature (Just et al., 2015). The finely ground leaves (15 g) were mixed with sand (2 g) and placed into the portafilter of an espresso machine (BES81BSS; Breville) and horopito extract (c. 200 mL) was generated in a hot solution of 35% ethanol in water (vol/vol). The extraction was repeated on six separate 15 g batches of ground leaves. The combined extracts (c. 1.4 L) were evaporated to about half the total volume (c. 700 mL) under reduced pressure to remove ethanol. The resulting concentrated extract was then divided into three equal portions, and each portion was extracted with *n*-hexane (8 × 200 mL). The hexane fractions were combined, dried over $MgSO_4$, filtered with Whatman filter and evaporated under reduced pressure to yield a yellow viscous oil (2.6 g). Hexane (c. 10 mL) was added to the oil, which was left overnight in the fridge. A yellow precipitate of polygodial was obtained when seeded with a fresh sample of commercial polygodial, which was filtered and washed with c. 50 mL of cold *n*-hexane to yield a yellow fluffy solid. A second crop of crystals was obtained from the mother liquor as yellow fluffy solid, thus yielding a total of 785 mg (0.7% wt/wt) of polygodial. An NMR spectrum was recorded on Bruker AVANCE 400 spectrometer at 400 MHz (^1H) (Figure S2) and was in agreement with the literature (Just et al., 2015).

2.2.2 | Synthetic derivatives of polygodial (Figure S1; compounds 4 and 5)

Compound 4 (Drimendiol) (Just et al., 2015)

Extracted polygodial (0.5 g, 2.13 mmol) was dissolved in dry ethanol (12 mL) at 0°C under nitrogen, to which $NaBH_4$ (0.35 g, 9.32 mmol) was slowly added in small portions. The resulting mixture was stirred at 0°C for 30 min, which was followed by stirring at room temperature for 1 h under nitrogen. After evaporating ethanol, water (50 mL) was added, and the resulting solution was extracted with dichloromethane (DCM; 4 × 20 mL). The combined organic fractions were dried over $MgSO_4$, filtered and evaporated to yield Compound 4 as a pale-yellow oil (0.32 g, 63%).

Compound 5 (Manwill et al., 2020)

Extracted polygodial (2.1×10^{-4} mol, 50 mg) was dissolved in acetonitrile (2 mL). Sodium phosphate monobasic monohydrate (15 mg, 1.1×10^{-4} mol) and 30% H_2O_2 (22 µL, 9.4×10^{-4} mol) dissolved in water (0.2 mL) was added to the above solution. A solution of sodium chlorite (96 mg, 1.1×10^{-3} mol) in water (0.5 mL) was added dropwise over 15 min. The reaction was then stirred for a further 24 h at room temperature. Solid Na_2SO_3 (5 mg) was added and the mixture was stirred for 5 min, followed by acidification using 10% HCl. The product was extracted into DCM (3 × 10 mL). The organic layer was washed with brine (3 × 10 mL). The combined organic layer was dried over $MgSO_4$, filtered and evaporated to yield Compound 5 as a white solid (40 mg, 70% yield).

2.3 | Antimycelial activity assay

The general procedure reported by Hunter, McDougal, et al. (2018) was followed for compound screening with the exception that V8 medium was used in our assays. A range of concentrations (1–200 µg/mL) of the test compounds in V8 medium were prepared through two-fold serial dilutions of the stock solutions in the different wells of 24-well assay plates (MediRay; Corning). Mycelial agar plugs (3 mm diameter) taken from the edges of 7-day-old plates of all the *Phytophthora* species were carefully transferred into the wells containing the test compounds. Each plate had appropriate growth controls (mycelial plug without any compounds), sterility controls (medium only) and antibiotic controls (chlortetracycline). Three replicates were included in the assay and the plates were incubated at 24°C for 7 days. The assay was repeated on three different days. A typical plate layout of the antimycelial assay is shown in Figure S3. Generally, mycelia grew to the edge of the 24-well plates within 2–3 days after incubation (end point). To ensure mycelia had stopped growing in the presence of inhibitory compounds, we incubated the 24-well plates for 1 week. The minimum concentration that stopped mycelial growth after 1 week of incubation was inferred as the antimycelial concentration.

2.4 | Zoospore motility and germination assay

A protocol developed by Lawrence et al. (2017, 2019) was modified slightly to test the zoospore motility and germination. Briefly, 10 agar mycelial plugs (3 mm diameter) of *Phytophthora* species (*P. cinnamomi* NZFS 3910, *P. agathidicida* 18406 and *P. multivora* 20280)

were placed into an empty Petri dish, submerged in approximately 30 mL of 20% V8 medium and incubated at 24°C in the dark for 1 week to allow mycelial growth. Formation of sporangia, which house the zoospores, was induced by carefully decanting the V8 medium without disturbing the mycelia followed by submerging these in either (c. 50 mL) sterile pond water (*P. multivora*) or 5% filtered soil extract (*P. cinnamomi* and *P. agathidicida*) for 4 h. The liquid was then decanted, and the mycelial plugs again submerged either in fresh pond water or soil extract solution for a further 4 h. This procedure was repeated three times, followed by overnight submergence of the mycelial plugs in a lit area to induce sporangia formation. The newly formed sporangia were analysed under a light microscope (Eclipse; Nikon). To release the zoospores, the solution was carefully decanted without damaging the sporangia and replaced with sterile water followed by incubation at 4°C for 1 h. After this time, the Petri dishes containing the sporangia were left at room temperature to release the zoospores. On average 1000 zoospores/mL were released and collected. The zoospores were counted using a haemocytometer (Marienfeld).

2.4.1 | Motility

The ability of antifungal compounds to prevent the motility of zoospores was studied using light microscopy (40× magnification). Briefly, zoospore suspensions (1 mL, approximately 1000 zoospores) were transferred into the different wells of a 24-well plate. The test compounds (50 µL) were two-fold serially diluted to a concentration range of 1–200 µg/mL and added to the zoospore suspensions. The zoospores in the plates were observed under a light microscope for 5 min and the time taken for all the zoospores to become immobile recorded. Concentrations of the compounds at which the zoospores remained motile after 5 min of treatment were considered as having no effect on zoospore motility and therefore ineffective. The experiment was done in triplicate and repeated on three different days. Representative videos of zoospores in the presence (Video S1) and absence (Video S2) of Compound 3 are included in the supplementary information.

2.4.2 | Germination

Using stock solutions of the test compounds either prepared in 40% V8 medium or 1% dimethyl sulphoxide (DMSO) in 40% V8 medium, a series of dilutions (1–200 µg/mL; 100 µL each) were prepared in 96-well plates. Each well containing the test compounds was then inoculated with 100 µL of the zoospores. Growth and sterility controls were included on each plate. The plates were incubated at 24°C overnight. The minimum concentration required to visually (compared to the growth control wells) inhibit mycelial growth was taken as the minimum inhibitory concentration (MIC) for zoospore germination. The experiment was done in triplicate and was repeated on three different days.

2.5 | Antioospore activity

The protocol developed by Boutet et al. (2010) was followed to generate and extract *P. agathidicida* and *P. multivora* oospores. Briefly, 10 agar plugs from the edges of freshly grown *Phytophthora* plates were placed in an empty Petri dish and the plugs were submerged in 20% V8 medium (c. 30 mL). The plates were incubated in the dark at 24°C for up to 14 days to generate oospores (monitored under light microscopy). The mycelial mats containing the oospores were carefully excised and mixed with distilled water (10 mL) using a tissue homogenizer to form a suspension. The suspension was centrifuged (2958 × *g* for 20 min) and washed three times with Milli-Q water to obtain a pellet. The pellet was resuspended in glucanex solution (Sigma-Aldrich) at 5 mg/mL and incubated overnight at 20°C to dissolve any mycelial and sporangia fragments. This was again centrifuged (2958 × *g* for 20 min) to obtain a pellet and the supernatant containing the glucanex solution was decanted. The pellet was further washed with water and centrifuged to obtain the oospores. These oospore solutions were then resuspended in distilled water at a concentration of approximately 1.6×10^4 oospores/mL as determined using a haemocytometer.

The effect of antioomycete compounds on oospore viability was tested using a tetrazolium bromide staining assay following the same literature protocol (Boutet et al., 2010). The test compounds (100 µL) at concentrations ranging between 12.5 and 1000 µg/mL were incubated with the oospore suspension (100 µL, c. 1.6×10^4 oospore/mL) for 7 days at 20°C. The experiment also included growth and sterility controls. Oospores treated at 70°C for 2 h were used as the positive control. The treated oospores were stained with 0.1% (wt/vol) tetrazolium bromide (50 µL) and incubated for another 48 h at 36°C, which were then viewed under the light microscope. Oospores that stained pink were considered viable, whereas the black and unstained ones were considered nonviable. At each compound concentration, the cell viability was determined as a percentage by counting the number of viable and nonviable cells using a haemocytometer placed in the light microscope. The experiment was done in triplicate and was repeated on two different days.

2.6 | Scanning electron microscopy analysis of mycelia and oospores

To assess the morphology of *P. agathidicida* and *P. multivora* mycelia, test compounds at 200 µg/mL were added to the mycelia, which were excised from the agar plates and incubated at 24°C for 5 days. The treated mycelial suspensions were centrifuged at 7558 × *g* for 5 min, washed three times in phosphate-buffered saline (PBS) solution and the pellet resuspended in 2.5% glutaraldehyde in PBS overnight at 4°C. Glutaraldehyde solution was discarded, and the pellets were washed three times with PBS to remove residual glutaraldehyde. The mycelial pellets were dehydrated in graded ethanol series (30%, 50%, 70%, 90% and 100% twice) for 10 min each and dried at 37°C for 10 min to completely remove any traces of ethanol. The dried samples were placed in a carbon tape and sputter coated with

platinum for 40 s at 25 mA before viewing under high vacuum using a scanning electron microscope (SEM; Hitachi Su-70) at 10 kV.

The *P. agathidicida* oospores for SEM analysis were prepared following the same procedure as described in Section 2.5, which were then treated with the lipopeptide at 1000 μg/mL for 7 days at 20°C. Oospores heated for 2 h at 70°C were used as the control. The treated oospores were imaged as described above for the mycelia but at 5 kV.

2.7 | Efficacy analyses on kauri and avocado leaves

Kauri leaves were collected from a kauri tree (>20 years old) at Plant & Food Research. Avocado leaves were collected from a tree (c. 10 m in height) in a residential area in Auckland. The length of each leaf was measured from apex to base using a ruler. The leaf surface was disinfected with 1% vol/vol sodium hypochlorite for 1 min and washed twice in distilled water. The leaves were dried in a laminar flow hood for 1 h. A small incision (c. 2 mm) from 1 cm below the apex of the leaves was made using a sharp scalpel. Stock solutions of the test compounds were prepared (1–20 mg/mL) either in water (lipopeptide, chlortetracycline and Agri-fos 600 with 60% phosphite) or 3% vol/vol DMSO (polygodial) and 10 μL of these were placed onto the incisions. Leaves treated with water, 3% vol/vol DMSO, chlortetracycline or phosphite served as the controls in this experiment. All leaves were placed in a moisture chamber. A semicircular mycelial plug (3 mm diameter) taken from the edge of *P. agathidicida* or *P. cinnamomi* 21,050 plates was placed on the incision point of the kauri (*P. agathidicida*) and avocado (*P. cinnamomi* 21,050) leaves and these were incubated at room temperature for 14 days. After 14 days, the length of the lesion area on the leaves was measured and converted into percentage infection coverage. Sample size for each experiment was 10 leaves and the experiment was repeated on three different days.

2.8 | Phytotoxicity analyses on kauri and avocado leaves

The method described above for efficacy testing was followed to test the phytotoxicity of the compounds on kauri and avocado leaves with the exception that this time the leaves were not inoculated with *Phytophthora* plugs. All leaves were placed in a moisture chamber and incubated at room temperature for 14 days. After 14 days, the lesion length (characteristic of necrosis) of each leaf was measured and converted into percentage lesion coverage. A sample size of 10 leaves was used per experiment and the experiment was repeated on three different days.

2.9 | Efficacy analysis on soil infected with *P. agathidicida*

Phytophthora agathidicida suspension for this experiment was prepared using the same procedure as described in Section 2.5. After 14 days, carefully excised mycelial mats were weighed, suspended in distilled water (20 mL) and agitated using a tissue homogenizer to form a uniform suspension, which was further diluted to 300 mL to obtain a concentration of approximately 0.1 g/mL. Soil (Daltons Premium Potting Mix, 15 g) was tightly packed in plastic containers and infected with *P. agathidicida* (5 mL) suspension. Immediately after adding the *P. agathidicida* suspension, the soil was flooded with distilled water (150 mL) and left at room temperature for 24 h. Water was carefully drained from the infected soil, which was treated with a solution of the lipopeptide (5 mL, 0.2 mg/mL) and polygodial (5 mL in 1% vol/vol DMSO, 0.2 mg/mL). The infected soil treated with water (5 mL), DMSO (1% vol/vol, 5 mL), chlortetracycline (5 mL, 0.2 mg/mL) and phosphite (10% vol/vol, 5 mL) served as controls for this experiment. Uninfected soil sample was used as the sterility control. A total of five soil samples per treatment was used in the experiment and the experiment was repeated twice. The soil samples were left at room temperature for 4 weeks. During this period, the soil samples were kept moist. After 4 weeks, soil samples were carefully flooded with sterile Milli-Q water (150 mL). Each sample was baited by placing Himalayan cedar needles (five needles) and germinated blue lupin seedlings (two seedlings) on the surface of the water. Baits were left at room temperature for 7 days. After 7 days, the cedar needles and germinated lupins were removed, rinsed once with sterile Milli-Q water and soaked in 70% ethanol (30 s) and water (30 s), followed by blot drying on a paper towel. Per treatment, whole dried cedar needle baits (five needles) and germinated tubes of the lupin seedlings (two germinated tubes) were grown on *Phytophthora*-selective medium (PARPH) supplemented with the following antibiotics: ampicillin (250 mg/L), penta-chloronitrobenzene (100 mg/L), pimaricin (10 mg/L), rifampicin (10 mg/L) and hymexazol (25 mg/L) at 24°C in darkness for 3 days. After 3 days, any mycelial growth observed on the PARPH plates was plated onto V8 agar and the plates incubated at 24°C for further 5 days. The mycelial growth observed in the V8 agar was visualized under light microscopy.

3 | RESULTS

3.1 | Antimycelial activity of the compounds

The antimycelial activity of the compounds against five different *Phytophthora* isolates is summarized in Table 1. The synthetic antifungal peptides showed antimycelial activity in the 25–200 μg/mL range against the *Phytophthora* strains. Amongst the three peptides tested, Compound 3 showed the best antimycelial activity (<100 μg/mL) against four of the *Phytophthora* strains. Polymyxin B was inactive against four *Phytophthora* strains (MIC > 200 μg/mL) while its MIC against *P. multivora* was 50–100 μg/mL. Chlortetracycline exhibited potent (2.5–20 μg/mL) antimycelial activity against all *Phytophthora* strains, particularly against the mycelia of *P. agathidicida* and *P. cinnamomi*.

The two polygodial samples (extracted and commercial) included in this study were the most potent against mycelia (50 μg/mL). The

TABLE 1 Summary of antioomycete activity of compound library against *Phytophthora* strains.

Compound description	Origin	Antimycelial MIC (μg/mL)					Zoospore motility MIC (μg/mL)[a]			Zoospore germination MIC (μg/mL)			
		P. cinnamomi			*P. multivora*	*P. agathidicida*	*P. cinnamomi*	*P. multivora*	*P. agathidicida*	*P. cinnamomi*	*P. multivora*	*P. agathidicida*	
		20276	3910	21050	20280	18406	3910	20280	18406	3910	20280	18406	Oospores
Lipopeptide-based													
Compound 1	Synthetic	>200	>200	>200	50–100	>200	–	–	–	–	–	–	–
Compound 2	Synthetic	100–200	>200	100–200	50–100	50–100	–	–	–	–	–	–	–
Compound 3	Synthetic	50–100	50–100	100–200	25–50	50–100	4–8	5–10	4–8	25–50	12.5–25	12.5—25	Active
Polygodial-based													
Commercial polygodial	Synthetic	10–20	12.5–25	25–50	10–20	5–10	12.5–25	12.5–25	10–20	50–100	25–50	25—50	Not active
Polygodial extracted from horopito	Natural (plant)	10–20	12.5–25	25–50	10–20	5–10	12.5–25	12.5–25	10–20	50–100	25–50	25–50	-
Diol form (Compound 4)	Synthetic	100–200	100–200	100–200	100–200	100–200	–	–	–	–	–	–	–
Dicarboxylic acid form (Compound 5)	Synthetic	100–200	100–200	100–200	50–100	50–100	–	–	–	–	–	–	–
Polymyxin B	Natural (bacteria)	>200	>200	>200	50–100	>200	–	–	–	–	–	–	–
Chlortetracycline	Natural (bacteria)	2.5–5	5–10	10–20	5–10	2.5	–	–	–	–	–	–	Not active
Agri-fos 600	Synthetic	1%–2% vol/vol	1%–2% vol/vol	0.25%–0.5% vol/vol	0.25%–0.5% vol/vol	0.25%–0.5% vol/vol	–	–	–	–	–	–	–

Abbreviation: –, not tested.

[a]Zoospore motility was observed for 5 min. Inhibitory activity of the compound is defined as the minimum inhibitory concentration (MIC) required for all the zoospores to become immobile within the first 5 min of the assay.

diol (Compound 4) and dicarboxylic acid (Compound 5) versions of polygodial (Figure S1) showed approximately 10 times weaker antimycelial activity (50–200 μg/mL) than polygodial, although Compound 5 was two-fold more active than Compound 4 against *P. multivora* and *P. agathidicida* strains.

Scanning electron microscope analysis of *P. agathidicida* and *P. multivora* mycelia treated with polygodial and Compound 3 showed morphological changes indicating damage to the mycelial surface as compared to the untreated mycelia (Figure S4a). Amongst the five strains included in this study, *P. multivora* appeared to be the most susceptible to the tested compounds. The antimycelial screening assays showed that polygodial is the most active antimycelial compound in this study, with Compound 3 (the longest peptide) also showing promising (<100 μg/mL) antimycelial activity. Therefore, polygodial and Compound 3 were selected for further analyses.

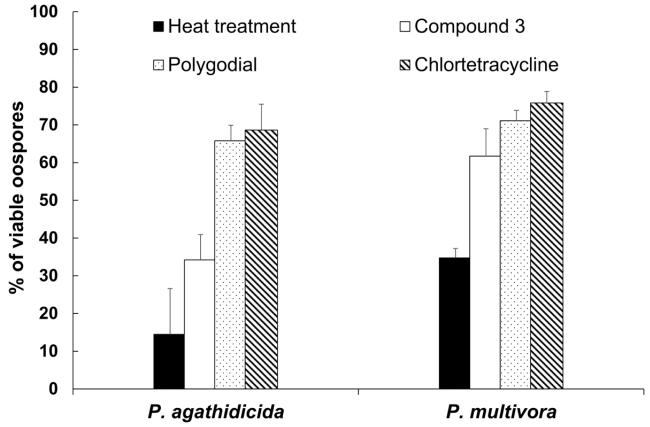


FIGURE 1 Percentage of viable oospores of *Phytophthora agathidicida* and *P. multivora* in the presence of different compounds at 1000 μg/mL and under heat treatment (heated at 70°C for 2 h).

3.2 | Antizoospore activity

The ability of Compound 3 and polygodial (commercial and extracted) to inhibit the motility and germination of *P. agathidicida*, *P. cinnamomi* 3910 and *P. multivora* zoospores were investigated (Table 1; Videos S1 and S2). Unfortunately, the other *P. cinnamomi* strains (*P. cinnamomi* 20276 and *P. cinnamomi* 21050) failed to produce sporangia and therefore could not be included in this assay.

Compound 3 was the most effective (4–10 μg/mL) at inhibiting the zoospore motility (5 min contact time) in all three strains. Compound 3 at a concentration of 25 μg/mL was enough to stop the complete movement of all the zoospores in all tested *Phytophthora* strains in less than 1 min (Video S1). Polygodial, although at a slightly higher concentration (10–25 μg/mL) than Compound 3, also inhibited zoospore motility of the three tested strains. It was also slower, requiring up to 5 min to completely stop the motility of all zoospores at 10–25 μg/mL.

Compound 3 was also the most effective (12.5–50 μg/mL) in preventing zoospore germination in *P. agathidicida*, *P. cinnamomi* 3910 and *P. multivora*. Polygodial (commercial and extracted) prevented zoospore germination when used at 25–100 μg/mL. Based on these assays, it can be concluded that, from amongst the compounds included in this study, Compound 3 is the compound of choice for inhibiting zoospore motility and preventing zoospore germination.

3.3 | Effect on oospores

The efficacy of the compounds on *Phytophthora* oospores was investigated in comparison to heat treatment. The percentage of viable oospores and their morphology was analysed (Figure 1, Figure S4b).

Heat treatment was the most effective at reducing the oospore viability, resulting in only 14% (*P. agathidicida*) and 34% (*P. multivora*) viable oospores after the treatment (Figure 1). After treatment with Compound 3 at its highest concentration (1000 μg/mL), 34% (*P. agathidicida*) and 62% (*P. multivora*) of the oospores remained viable. Neither polygodial nor chlortetracycline at 1000 μg/mL had any effect on oospore viability of the two pathogens (Figure 1).

The SEM analysis showed that most of the untreated oospores retained their characteristic "balloon" shapes with fewer oospores exhibiting some structural distortion (Figure S4b). The oospores treated with Compound 3 were clearly different and showed more structural distortion. As expected, heat treatment was the most effective and caused a complete loss of structural integrity of the oospores, leading to cell lysis with extensive remnants scattered across (Figure S4b).

3.4 | Leaf assay

As evident from Figure 2, control kauri leaves (treated with water or 3% vol/vol DMSO) showed 51% and 44% surface coverage by *P. agathidicida* (Figure 2). Kauri leaves treated with the polygodial, Compound 3 and the antibiotic chlortetracycline, on the other hand, showed only 0%–10% of surface coverage by the pathogen. Results of the detached leaf assays revealed that all our inhibitory compounds (at 20–200 μg) protected kauri leaves from *P. agathidicida* infection (Figure 2) and avocado leaves from *P. cinnamomi* (Figure S5) for up to 14 days.

The phytotoxicity analyses showed that the lesion coverage after treatment with Compound 3 on kauri leaves was only 1% (Figure S6). Treatment with chlortetracycline showed 15% lesion coverage on kauri leaves. DMSO (control) showed 43% lesion coverage, while the water-treated samples (control) had no lesions. Some symptoms of phytotoxicity could be seen in the polygodial-treated leaves (37% lesion coverage), which we believe is caused by traces of DMSO that was used to solubilize polygodial. Phosphite at 20% vol/vol concentration (1200 μg) was significantly phytotoxic to kauri leaves (59% lesion coverage).

Compound 3, polygodial and chlortetracycline treatments did not show noticeable necrosis or lesions and therefore can be

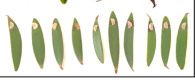
Treatment	Images of leaves	Leaf length (mm)	Infected leaf length (mm)	Infection coverage (%)
Uninfected control		73 ± 13	0	0
Water		70 ± 9	36 ± 7	51 ± 10
DMSO (3%)		70 ± 7	31 ± 10	44 ± 14
Polygodial (20 μg)		66 ± 8	3 ± 7	5 ± 11
Compound 3 (200 μg)		73 ± 6	7 ± 10	10 ± 13
Chlorotetracycline (20 μg)		74 ± 5	0	0

FIGURE 2 Kauri leaves infected with *Phytophthora agathidicida* mycelia treated with water or the inhibitory compounds listed at the given quantities. Values are means ± SD.

concluded as having negligible to low phytotoxicity on kauri and avocado leaves (data not shown).

The results from the detached leaf assays have shown that polygodial and Compound 3 at the higher concentrations tested are nonphytotoxic and at the lower tested concentrations protect kauri leaves from *P. agathidicida* and avocado leaves from *P. cinnamomi* infections.

3.5 | Soil assay

The ability of the anti-*Phytophthora* compounds to target *P. agathidicida* in infected soil was undertaken and the results are summarized in Figure 3 and Figure S7. Mycelial growth from the original *P. agathidicida* 18406 strain and from the uninfected soil sample are shown in Figure 3a,b, respectively. Mycelial growth (Figure S7) and the extent of coverage observed on V8 agar plates by *Phytophthora* isolated from either floating cedar needles or germinated tubes of the lupin seedlings of the control soil samples (water, Figure 3c) and 1% vol/vol DMSO (Figure 3d) were similar to those from the original *P. agathidicida* 18406 strain (Figure S7, Figure 3a). However, when plated out, no *Phytophthora* was isolated from the floating baits of the soil sample treated with polygodial (Figure 3g, Figure S7g), indicating that polygodial is capable of targeting and inactivating *P. agathidicida* in infected soil. Treatment with phosphite (Figure 3f) showed almost no mycelial growth (Figure S7f) from the isolated baits. When plated out, the growth of *Phytophthora* isolated from baits of the soil samples treated with chlortetracycline (Figure 3e) and Compound 3 (Figure 3h) was similar to the growth observed in the control samples (water and 1% vol/vol DMSO), implying that these treatments failed to target and inactivate *P. agathidicida* in infected soil.

4 | DISCUSSION

Anti-*Phytophthora* compounds active against multiple life stages of *Phytophthora* pathogens are scarce. Commercial pesticides commonly used against *Phytophthora* pathogens suffer from several drawbacks (Dick & Kimberley, 2013; Dobrowolski et al., 2008; Horner et al., 2015; Miao et al., 2020). Thus, novel compounds, particularly those target multiple life stages of *Phytophthora* pathogens, are urgently needed. Our current strategy to develop novel anti-*Phytophthora* compounds relies on the use of known antifungal compounds of synthetic (De Zoysa et al., 2018) and natural (McCallion et al., 1982; Wayman et al., 2010) origin to target mycelial, zoospore and oospore life stages of several *Phytophthora* pathogens.

The synthetic antifungal peptides (Compounds 1–3) included in this study vary in peptide chain length and therefore the net cationic charge (De Zoysa et al., 2018). Our previous results indicated that the higher the net cationic charge, the more potent the peptides were against fungi (De Zoysa et al., 2018). In the current study, these antifungal peptides were investigated to establish whether they have any antimycelial activity against *Phytophthora* species. Results from the antimycelial assay revealed that Compound 3, with the highest cationic charge, is the most active lipopeptide against the mycelial life stages of the *Phytophthora* species, indicating that a higher overall cationic charge increases the potency of these peptides against *Phytophthora* species, as was observed previously with *C. albicans* (De Zoysa et al., 2018).

The SEM images of *P. agathidicida* and *P. multivora* treated with Compound 3 showed cellular remnants scattered across the mycelial surface. This was more prominent in *P. multivora*, which is not surprising because *P. multivora* was slightly more sensitive to Compound 3. Leakage of intracellular content because of membrane lysis is widely documented in the literature for membrane lytic antimicrobial lipopeptides, as has also been reported in our own previous research involving both bacterial and fungal pathogens (De Zoysa et al., 2015, 2018). The current results from SEM analyses establish that Compound 3 also causes membrane perturbation on the mycelia of *Phytophthora* pathogens.

Polygodial exhibited the best antimycelial activity against all the tested *Phytophthora* pathogens. Several synthetic polygodial analogues have been developed to improve the biological properties of polygodial. Most of these analogues have been designed by

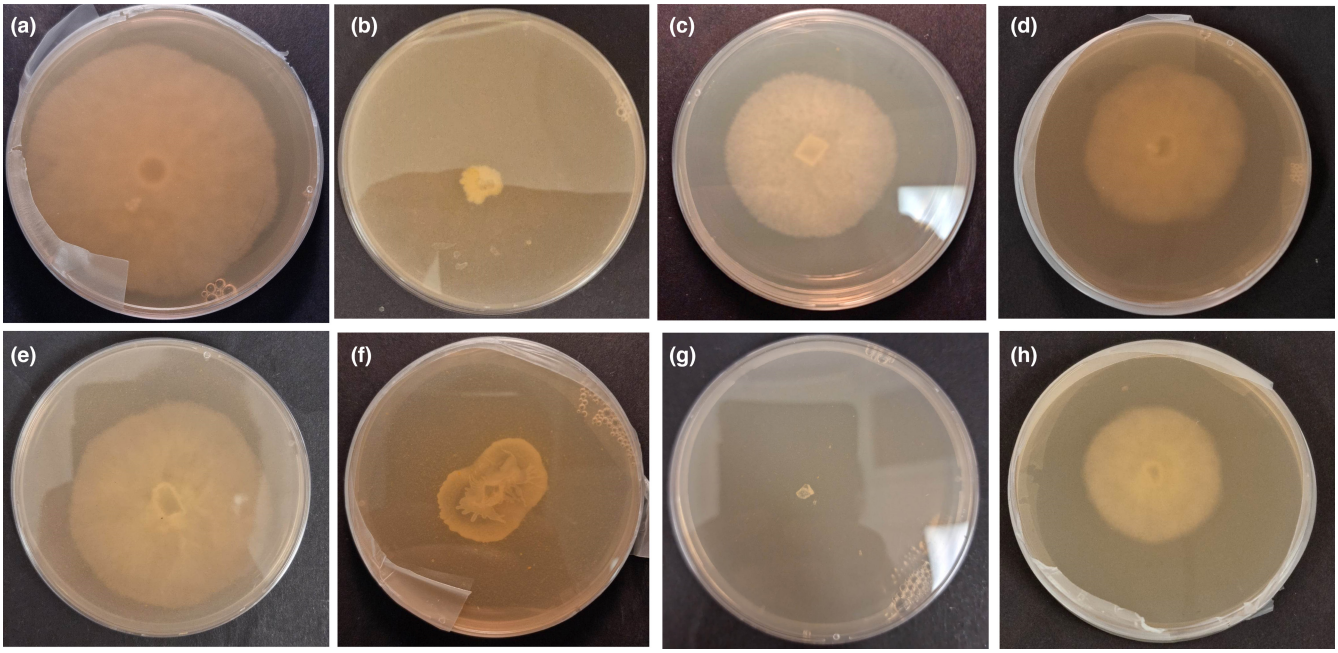


FIGURE 3 Mycelial growth observed on V8 agar from the soil-baiting experiment. (a) Original *Phytophthora agathidicida* 18406 mycelia, (b) uninfected soil sample; treatments used: (c) water, (d) 1% vol/vol dimethyl sulphoxide (DMSO), (e) chlortetracycline, (f) phosphite, (g) polygodial and (h) Compound 3.

replacing the dialdehyde moiety of the drimane sesquiterpene scaffold with other reactive functional groups (Derita et al., 2013; Manwill et al., 2020). In our investigations, the reduced (Compound 4) and oxidized (Compound 5) forms of polygodial showed a 10-fold reduction in antimycelial activity compared to polygodial itself. These results indicate that the dialdehyde moiety of the drimane sesquiterpene scaffold is crucial for its antimycelial activity and should not be altered when designing synthetic analogues against *Phytophthora* species.

The SEM images of *P. agathidicida* and *P. multivora* mycelia treated with polygodial and chlortetracycline also showed damages to mycelial structure, which looked visibly unhealthy, as compared to the control. Chlortetracycline is known to initiate antimicrobial activity by targeting protein synthesis. Polygodial's antifungal activity has been attributed to two distinct mechanisms, one where it acts as a nonionic surfactant disrupting the cellular membrane thus inducing cell leakage (Kubo et al., 2001) and the other through acting on internal targets such as the vacuoles (Kondo et al., 2016) or causing mitochondrial ATPase inhibition (Lunde & Kubo, 2000). Further experiments such as transmission electron microscopy and membrane staining assays will be required to validate the actual mechanism of polygodial against *Phytophthora*.

Compound 3 emerged as the most effective at targeting *Phytophthora* zoospores. Generally, the zoospores released from sporangia under cold shock conditions are very short-lived, although they can swim up to several centimetres at a speed of 200 μm/s to infect root tips of new hosts (Hardham & Blackman, 2018). Colonization of a new host is then initiated by zoospore encystment and germination where the flagella are either removed or retracted, depending on the species, followed by the rapid production of the cell wall. Therefore, it is imperative that the antimicrobial agent targeting these zoospores should exhibit fast-killing kinetics to immobilize the short-lived mobile zoospores and prevent them from infecting new hosts. Antimicrobial lipopeptides possess rapid kill kinetics because of their nonspecific action on cell membranes. This property of rapid kill kinetics was also evident in the current study, where *Phytophthora* zoospores became completely immotile within 1 min of the addition of Compound 3. Consequently, Compound 3 prevented zoospore germination at a slightly lower concentration than polygodial. Longer contact time and a higher concentration were required to stop zoospore motility using polygodial. Polygodial may be exerting its antizoospore activity through interaction with similar intracellular targets. However, further experiments such as transmission electron microscopy will be needed to corroborate this hypothesis.

Oospores are crucial for the long-term survival of *Phytophthora* (Hardham & Blackman, 2018). Additionally, their thick cell walls help them withstand harsh environmental conditions and remain resistant to chemical treatments such as sterigene (Bellgard et al., 2010; Dick & Kimberley, 2013). To the best of our knowledge, only disinfectants such as Janola (sodium hypochlorite, active ingredient; Bellgard et al., 2010; Dick & Kimberley, 2013), Virkon S (dipotassium peroxodisulphate; Bellgard et al., 2010; Dick & Kimberley, 2013) and the antibiotic oxathiapiprolin (Lacey et al., 2021) have shown any noticeable activity against oospores of *P. agathidicida*. Unfortunately, these products have limited scope in *Phytophthora* control due to phytotoxicity (Bellgard et al., 2010) and the development of resistance (oxathiapiprolin; Miao et al., 2020). Heat treatment of *P. agathidicida* oospores either at 55°C over 4 h or at 70°C over 30 min has also been shown to reduce oospore viability (Dick & Kimberley, 2013). However, heat treatment is not a viable option that can be applied in the field. Results of the present investigations indicate that although

less effective than heat treatment, Compound 3 at 1000 μg/mL does reduce the viability of oospores, making it a welcome addition to the very limited number of treatment options available to target *Phytophthora* oospores. SEM images of *P. agathidicida* oospores treated with Compound 3 showed damage to the thick cell walls, validating its potential to be further developed as an effective treatment against *Phytophthora* oospores.

The main aim of this study was to screen and identify new anti-*Phytophthora* compounds and gather preliminary data to evaluate their viability for future seedling experiments. Detached leaf assays are rapid and standardized to determine the ability of compounds to protect host plant organs from infection. These assays have been used in the literature to study the infectivity of soilborne *Phytophthora* species such as *P. agathidicida* (Herewini et al., 2018; Horner & Hough, 2014; Lacey et al., 2021), *P. medicaginis* (Irwin et al., 2003) and *P. ramorum* (Balci et al., 2008). The recent study by Lacey et al. (2021) showed that oxathiapiprolin delays the onset of *P. agathidicida* infection on detached kauri leaves depending on the concentration. This study has shown that both polygodial and Compound 3 provide noticeable protection to kauri and avocado leaves from *Phytophthora* infections without inducing leaf phytotoxicity. The next step in our investigations will be to validate the ability of polygodial and Compound 3 to protect seedlings from infections.

Preliminary efficacy studies to test whether polygodial and Compound 3 can target and deactivate *P. agathidicida* in soil were undertaken using a soil-baiting experiment. Results from these experiments indicate that polygodial inhibited the growth of *P. agathidicida* in soil. It is reasonable to conclude that the low molecular weight (234 Da) of polygodial allows its efficient diffusion through the soil to target the pathogen and deactivate it. However, chlortetracycline and the lipopeptide, Compound 3, failed to target and deactivate *P. agathidicida* in soil. Tetracycline antibiotics such as chlortetracycline have been reported to be readily adsorbed onto the soil with high organic matter (Conde-Cid et al., 2019), which may explain the observed lack of efficacy of chlortetracycline in the soil-baiting experiment. The inability of the lipopeptide to target *P. agathidicida* in soil could be attributed to its poor diffusion capacity in soil due to its larger molecular weight (2.7 kDa) and soil adsorption. Recent studies suggest that polymyxin B and E (colistin) can be adsorbed into different types of soil (Davis & Janssen, 2020; Peng et al., 2022). The structural similarity of Compound 3 to polymyxins leads us to believe that it would have a similar soil-adsorption profile to the polymyxins. Further experiments will be essential to establish the soil-adsorption property of Compound 3, which should then be followed by techniques such as the development of suitable formulations to render it useful in the field.

This study has shown that antimicrobial agents with proven antifungal activity have the potential to target multiple life stages of aggressive *Phytophthora* species. Naturally derived and biodegradable anti-*Phytophthora* compounds are important in finding environmentally friendly solutions in the battle against diseases caused by *Phytophthora*. This study is the first report on the use of peptide-based therapeutics to target the different life stages (mycelial, zoospores and oospores) of *Phytophthora* species. Both polygodial and lipopeptide prevented *Phytophthora* infections on leaves without inducing leaf phytotoxicity. Preliminary results have shown that polygodial is capable of targeting *P. agathidicida* in infected soil. This study has demonstrated that antifungal lipopeptides such as Compound 3 and small molecule natural antifungal products such as polygodial could be potentially developed as biodegradable treatment options to combat *Phytophthora* pathogens. In order to establish the efficacy of these compounds as novel treatment options, we will conduct experiments on seedlings. These experiments will probably determine an appropriate route for the administration of these compounds. In addition, the compound library will be further extended against *P. infestans*, which is known to cause potato and tomato blight, and *P. pluvialis*, which infects radiata pine causing red needle cast disease. Investigations on the selectivity of these compounds to pathogenic *Phytophthora* species will also form part of our future study because it is important to ensure that beneficial soil bacteria are not killed by applying these compounds in the soil.

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CONFLICT OF INTEREST STATEMENT

The authors declare that there is no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available in the supplementary material of this article.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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