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1 # Assessing the effectiveness of oxathiapiprolin towards *Phytophthora*
2 # *agathidicida*, the causal agent of kauri dieback disease

3

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11
12 ## Abstract

13

14 *Phytophthora* species cause disease and devastation of plants in ecological and
15 horticultural settings worldwide. A recently identified species, *P. agathidicida*, infects and
16 ultimately kills the treasured kauri trees that are endemic to New Zealand. Currently
17 there are few options for controlling or treating *P. agathidicida*. In this study, we sought
18 to assess the toxicity of the oomycide oxathiapiprolin against several lifecycle stages of
19 two geographically distinct *P. agathidicida* isolates. Half maximal effective concentration
20 (EC_{50}) values were determined to be approximately 0.1 ng/ml for inhibiting mycelial
21 growth, indicating that *P. agathidicida* mycelia are more sensitive to oxathiapiprolin than
22 those from most other *Phytophthora* species that have been studied. Oxathiapiprolin
23 was also highly effective at inhibiting the germination of zoospores (EC_{50} = 2-9 ng/ml for
24 the two isolates) and oospores (complete inhibition at 100 ng/ml). In addition,
25 oxathiapiprolin delayed the onset of detached kauri leaf infection in a dose-dependent
26 manner. Collectively, the results presented here highlight the significant potential of
27 oxathiapiprolin as a tool to aid in the control of kauri dieback disease.

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28 **Introduction**

29

30 Members of the oomycete genus *Phytophthora* cause disease and destruction to
31 plants in agricultural and natural ecosystems worldwide (Hansen et al. 2012). For
32 example, it has been estimated that the infamous causal agent of potato late blight,
33 *Phytophthora infestans*, continues to cause >$6 billion of damage yearly (Haverkort et
34 al. 2008). In New Zealand, a recently identified species named *P. agathidicida* is
35 threatening kauri (*Agathis australis*), which are treasured, long-lived native conifers
36 (Bradshaw et al. 2020; Weir et al. 2015). Kauri are giant trees – the largest, Tāne
37 Mahuta, has a trunk girth of over 13 m – and they have a vital ecological role as
38 foundation species in the forests they inhabit (Wyse et al. 2014). *P. agathidicida* infects
39 kauri of all ages *via* the roots, causing trunk lesions, canopy thinning, root and collar rot,
40 and ultimately death (Bellgard et al. 2016). While kauri dieback disease was first
41 documented in the 1970s, it has spread rapidly in the past decade and now poses a
42 significant long-term threat to the species (Black et al. 2018; Waipara et al. 2013).
43 Currently, there are few options for controlling or treating *P. agathidicida*. The
44 main tool for reducing pathogen spread is physical barriers such as walking track
45 closures and shoe cleaning stations. The only chemical treatment that has been used in
46 the field is phosphite (phosphorous acid). In trunk injection field trials, phosphite
47 improved canopy health, and reduced lesion activity and expansion (Horner et al. 2015).
48 However, potential phytotoxic effects (Bradshaw et al. 2020; Horner et al. 2015) and the
49 potential for resistance to develop (Dobrowolski et al. 2008; Hao et al. 2020) are
50 concerns regarding phosphite treatment. Other chemical controls have been explored
51 (Lawrence et al. 2017), including surveying native New Zealand plants for their
52 production of oomycides (Lawrence et al. 2019), but none of these options has been
53 field tested to date.
54 Oxathiapiprolin is a first-in-class piperindinyl thiazole isoxazoline oomycide
55 (Pasteris et al. 2016) that is highly effective against many oomycete species when
56 applied preventatively or curatively (Belisle et al. 2019; Bittner et al. 2017; Cohen 2015;
57 Cohen et al. 2018; Gray et al. 2018; Humann et al. 2019; Ji and Csinos 2015; Miao et al.

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58 2016b; Miao et al. 2016a; Qu et al. 2016a). It can be applied *via* foliar sprays, soil
59 applications (*e.g.* in-furrow) or soil drenches and it is efficiently translocated throughout
60 plant tissues in a variety of horticultural species (Cohen 2015, 2020; Qu et al. 2016b),
61 including perennial tree crops (Gray et al. 2020). In addition, oxathiapiprolin has low
62 phytotoxic effects in host tissue. For example, in downy mildew infected sunflower, the
63 highest tested concentrations of oxathiapiprolin were effective in treating disease while
64 also causing no phytotoxic effects (Humann et al. 2019).
65 The intracellular target of oxathiapiprolin is an oxysterol binding protein (OSBP)-
66 related protein (ORP) (Bittner et al. 2017; Miao et al. 2018; Miao et al. 2016b; Pasteris et
67 al. 2016). While the precise function of ORPs in oomycetes is unknown, this family of
68 proteins exists throughout eukaryotes and its members are involved in a broad range of
69 functions including intracellular sterol transport, lipid metabolism, and signal transduction
70 (Raychaudhuri and Prinz 2010).
71 In this study, we have annotated the sequence of the *P. agathidicida* ORP1 gene
72 (*PaORP1*). In two geographically distinct isolates of *P. agathidicida*, we have assessed
73 the efficacy of oxathiapiprolin against different stages in the lifecycle including mycelial
74 growth, and both motility and germination of the infectious zoospores. By designing a
75 protocol to purify the metabolically dormant survival spores (oospores), we were also
76 able to test the efficacy of oxathiapiprolin against this life cycle stage, for the first time for
77 any *Phytophthora* species. Overall, our work establishes a baseline that strongly
78 supports field testing of oxathiapiprolin for the management of kauri dieback disease.
79

80 **Materials and Methods**

81

82 *Materials*

83

84 *P. agathidicida* isolates NZFS 3770 and NZFS 3772 were obtained from the
85 culture collection held at Scion (Rotorua, New Zealand). *P. agathidicida* 3770 was
86 originally isolated from Coromandel, New Zealand, while 3772 was isolated near
87 Auckland, New Zealand (Studholme et al. 2016). Oxathiapiprolin (≥98% purity) was

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88 purchased from Carbosynth (Compton, Berskhire, UK). It was stored at 1 mg/ml in 100%
89 dimethyl sulfoxide (DMSO) at -20 °C. To account for possible solvent effects, DMSO
90 concentrations were standardized and DMSO-only controls were performed in all
91 experiments. Pimaricin (2.5%, w/v, aqueous solution), rifampicin,
92 pentachloronitrobenzene, β-sitosterol and fluorescein diacetate were from Sigma
93 Chemical Co. (St. Louis, MO, USA). Ampicillin was from GoldBio (St. Louis, MO, USA).
94 TOTO-3 iodide was from Invitrogen. Brightfield microscopy was performed using an
95 Olympus CKX53 inverted light microscope with Olympus cellSens Standard software for
96 image processing.
97
98 *Bioinformatics*
99
100 The *ORP1* gene sequences from *P. capsici*, *P. infestans*, *P. ramorum* and *P.*
101 *sojae* have been published in the patent literature (Andreassi et al. 2013). Each was
102 used as the query sequence for a pairwise alignment with the *P. agathidicida* 3772 draft
103 genome (Studholme et al. 2016) using Parasail 2.4.1 (Daily 2016), implemented within
104 SnapGene 5.0.7. A candidate transcriptional start site and a likely intron were annotated
105 using the *Phytophthora* consensus sequences described previously (Kamoun 2003).
106 The translated sequence of the *Pa*ORP1 protein was analyzed using ProtParam
107 (Gasteiger et al. 2005), and the domains were identified using the NCBI Batch
108 Conserved Domain Search tool (Lu et al. 2020).
109
110 *Routine culture conditions*
111
112 *P. agathidicida* was cultured at 22 °C in the dark, unless otherwise noted. *P.*
113 *agathidicida* isolates were initially cultured on selection plates comprising cornmeal agar
114 (BD Difco; 17 g/l) supplemented with pimaricin (0.001%, w/v), ampicillin (250 μg/ml),
115 rifampicin (10 μg/ml) and pentachloronitrobenzene (100 μg/ml). A 6 mm diameter
116 section of the leading edge of mycelia was transferred to a clarified V8 agar plate.

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117 Clarified V8 agar was prepared by mixing 200 ml of Campbell's V8 juice with 2 g $CaCO_3$
118 on a magnetic stirrer for 10 min at room temperature. Next, it was clarified by
119 centrifugation at 7000*g* for 10 min. The clarified juice (200 ml) was added to 800 ml of
120 distilled, deionized water (ddH_2O) with 15 g of bacteriological grade agar (Formedium,
121 Hunstanton, UK) and sterilized by autoclaving. The isolates were then maintained by
122 routinely transferring them to new clarified V8 agar plates for the duration of the study.
123
124 *Mycelial growth inhibition testing*
125
126 Mycelial growth inhibition was measured on potato dextrose agar plates (BD
127 Difco; 39 g/l). Initially, 6 mm diameter agar plugs were taken from the leading edge of
128 mycelial growth on clarified V8 plates and transferred to potato dextrose plates
129 amended with varying concentrations of oxathiapiprolin. The plates were then incubated
130 at 22 °C in the dark for five days, with images subsequently taken of each plate. For
131 each replicate, six radial measurements were taken from the centre of the outermost
132 point of growth and averaged. The concentration of oxathiapiprolin required to effect
133 50% growth inhibition (the EC_{50}) was calculated using GraphPad Prism Version 8 by
134 plotting log-transformed oxathiapiprolin concentration against the average radius of
135 growth.
136
137 *Zoospore production and testing*
138
139 To produce zoospores, ten 6 mm diameter agar plugs were taken from the
140 leading edge of mycelial growth on clarified V8 agar plates using a cork borer and added
141 to 15 ml of 2% (w/v) carrot broth amended with 15 μg/ml β-sitosterol in 90 mm Petri
142 dishes. For 1 l of 2% (w/v) carrot broth, 20 g of frozen carrots were blended for 30 s in
143 500 ml of ddH_2O. The resulting mixture was then filtered through four layers of miracloth
144 (Merck). The volume was brought up to 1 l with ddH_2O and sterilized by autoclaving. The
145 dishes containing agar plugs were incubated in the dark for 30 h at 22 °C. The carrot

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146 broth was then removed and the mycelial mats were washed with 15 ml of a sterile soil
147 solution for 45 min. This had been prepared by stirring a 2% (w/v) solution of topsoil in
148 ddH_2O on a magnetic stirrer for 4 h at room temperature. After stirring, the solution was
149 left to settle overnight. The solution was filtered by passage through four layers of
150 miracloth (Merck) and then Whatman Grade 1 filter paper, before being sterilized by
151 autoclaving. After two washes, the mycelial mats were incubated in a further 15 ml of the
152 soil solution under continuous light for 14 h at 22 °C. The soil solution was then removed
153 and the mycelial mats were washed with 15 ml of sterile water for 10 min. This water
154 was removed and 15 ml of sterile water, pre-chilled to 4˚C, was added. The plates were
155 incubated at 4 °C for 20 min to stimulate zoospore release. The plates were stored at
156 room temperature until sufficient numbers of zoospore were released (typically 1-2 h).
157 Zoospores were pooled and counted using 2-chip disposable hemocytometers (Bulldog
158 Bio, Portsmouth, NH, USA).
159 For motility and germination testing, zoospores were first diluted to a standard
160 concentration of 5000 zoospores/ml. Next, 990 μl of this suspension was added to 24-
161 well plates containing 10 μl of oxathiapiprolin at 100× the desired final concentration. For
162 motility assays, zoospores were observed using brightfield microscopy at time intervals
163 of 0, 10, 20, 30, 60, 90, 120, 150, 180, 210, and 240 min, or until complete loss of
164 movement was observed. Following 240 min of observation, the 24-well plates were
165 stored at 22˚C overnight in the dark to assess germination. The following morning,
166 zoospore germination was measured by counting 25 randomly-selected zoospores per
167 well, with germinated spores being those with germ tubes at least twice the diameter of
168 the spore. EC_{50} values for germination were calculated using GraphPad Prism Version
169 8. The total number of germinated spores was plotted against the log-transformed
170 concentration of oxathiapiprolin.
171
172 *Oospore isolation and testing*
173

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174 For oospore production, three plugs (~3 mm diameter) were taken from the edge
175 of actively growing mycelia and transferred to a 90 mm Petri dish containing 15 ml
176 oospore growth medium (4% (w/v) carrot broth filtered through Whatman Grade 1 filter
177 paper and supplemented with 12 μg/ml β-sitosterol prior to autoclaving). The cultures
178 were incubated in the dark for 4-6 weeks at 22 °C. The resulting mycelial mats were
179 then transferred using sterile tweezers to 50 ml tubes and suspended in 40 ml of sterile
180 water. To separate the oospores from mycelia, the solution was homogenised for 1 min
181 (D160 Tissue Homogeniser, DLAB), then sonicated at 20% amplitude for 1 min (500
182 Watt Ultrasonic Cell Disruptor with a 5 mm probe, Sonics & Materials, Inc) and filtered
183 through 100 μm and 40 μm EASYstrainers (Greiner). The filtered oospore suspension
184 was pelleted at 1200*g* for 10 min, the supernatant removed, and the oospore pellet was
185 resuspended in 5 ml of sterile water. Oospore numbers were estimated using disposable
186 C-Chip haemocytometers (Bulldog Bio). Purified oospores were stored at 4 °C in the
187 dark.

188 Germination assays were set up in 96-well plates with ~1000 oospores per well.
189 A kauri root extract was used to stimulate germination. To prepare the extract, kauri
190 roots were harvested, finely ground in a blender and added to water at 10% (w/v). This
191 suspension was incubated overnight at room temperature with constant mixing. The
192 resulting liquid was sterilized by passing through a 0.22 μm syringe-driven filter.
193 Germination assays contained 10 μl of this kauri root extract, along with oxathiapiprolin
194 at varying concentrations, in a total volume of 100 μl. Control wells included the same
195 concentration of DMSO as sample wells. The plates were incubated under continuous
196 light for 5 days at 22 °C. Oospores were imaged using brightfield microscopy at 40×
197 combined magnification.

198 Oospore viability assays were carried out as we have described in detail
199 elsewhere (Fairhurst and Gerth 2021). Oxathiapiprolin at the indicated concentration
200 was added to ~2500 oospores in a total volume of 100 μl. The treated oospores were
201 incubated at 22 °C in the dark for 2 days prior to live/dead staining. Oospores were then
202 pelleted at 1200*g* for 10 min and the supernatant was removed. Next, 10 μl of

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203 fluorescein diacetate (live stain; 200 μM) was added to the oospores and incubated at
204 37°C in the dark for 20 h. Then 10 μl of TOTO-3 iodide (dead stain; 20 μM) was added
205 and the oospores further incubated at 37°C, in the dark for 4 h. Stained oospores were
206 imaged using a fluorescence microscope (Olympus BX63) at 40× magnification using
207 the green filter for fluorescein (excitation 465-495 nm, emission 515-555 nm) and red
208 filter for TOTO-3 iodide (excitation 540-580 nm, emission 590-665 nm). As a control for
209 the live/dead staining and image analysis, oospores were rendered non-viable by heat
210 treatment (98 °C, 24 h) before being stained and analyzed in parallel with the
211 oxathiapiprolin-treated samples. Oospores were automatically counted by analyzing the
212 images with CellProfiler v3.1.8 (McQuin et al. 2018) and the data were further processed
213 and visualized as described elsewhere (Fairhurst and Gerth 2021).

214

215 *Detached leaf assays*

216

217 Using a sterilized scalpel, a 2 mm incision was made 1 cm from the base of each
218 freshly harvested kauri leaf. Three leaves were placed in a 90 mm Petri dish and each
219 was spray coated with 200 μl of oxathiapiprolin at the indicated concentration. A 6 mm
220 diameter agar plug of *P. agathidicida* was taken from the leading edge of mycelial
221 growth and placed over the incision on each leaf. The plates were stored at 22 °C in a
222 12 h light : 12 h dark cycle with a dampened paper towel to prevent leaf drying. The
223 leaves were imaged daily for 10 days. The length of lesion spread was measured using
224 ImageJ (https://imagej.nih.gov/ij/).

225

226 **Results**

227

228 *Gene annotation and protein domain analysis*

229

230 In *Phytophthora*, the biochemical target of oxathiapiprolin is the ORP1 protein
231 (Bittner et al. 2017; Miao et al. 2018; Miao et al. 2016b; Pasteris et al. 2016). Before

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232 beginning our experimental studies, we endeavoured to identify the ORP1 ortholog in *P.*
233 *agathidicida*. An unannotated, partly-assembled genome for *P. agathidicida* isolate
234 NZFS 3772 has been reported (Studholme et al. 2016). Simple pairwise alignments
235 identified a highly conserved ortholog of 2,973 bp contained entirely within contig 1930
236 of the draft genome sequence.

237 A previous survey identified a conserved 16 bp motif (GCTCATTYBNNNWTTY)
238 surrounding the transcriptional start site of many oomycete genes, typically 50-100 bp
239 upstream of the start codon (Kamoun 2003). The *P. agathidicida ORP1* gene (*PaORP1*)
240 has a near-perfect match to this consensus (GAGCACTCGGCCTTTC; mismatches
241 underlined) located 153 bp upstream of the predicted start codon. In the same survey, it
242 was also noted that introns are relatively rare in *Phytophthora* genes, but that there are
243 conserved sequences at the 5' and 3' exon-intron junctions (5'-GTRNGT…YAG-3') and
244 a conserved intronic motif (CTAAC) important for splicing (Kamoun 2003). These
245 features clearly define an intron sequence of 90 bp in *PaORP1*, the splicing of which
246 also removes two in-frame termination codons. Thus, *PaORP1* encodes a 960-residue
247 protein. The annotated sequence of *PaORP1* is shown in Supplementary Fig. S1.

248 The *Pa*ORP1 protein is estimated to have a molecular weight of 104.4 kDa and
249 an isoelectric point (pI) of 6.5. Across the eukaryotes, ORP proteins are identified by a
250 signature motif (canonically EQVSHHPP), which in turn is found within the highly-
251 conserved OSBP-related domain (ORD) (Raychaudhuri and Prinz 2010). In the ORP1
252 proteins from *P. infestans*, *P. capsici* and *P. sojae*, the signature motif is EHTSHHPP
253 (Andreassi et al. 2013; Miao et al. 2018). *Pa*ORP1 has the same EHTSHHPP sequence
254 as these other *Phytophthora* orthologs and it is found at residues 728-735
255 (Supplementary Fig. S1). Also similar to ORP1 proteins from other *Phytophthora*
256 species (Andreassi et al. 2013), *Pa*ORP1 is predicted to have a pleckstrin homology
257 (PH) domain at its N-terminus, followed by a StAR-related lipid transfer (START)
258 domain. The third domain in the primary sequence is the ORD, which is 95-97%
259 identical to those from the other *Phytophthora* species we analyzed. The overall domain
260 organisation of *Pa*ORP1 is shown in Fig. 1.

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261 Altogether, these bioinformatic analyses confirmed that *P. agathidicida* contains
262 the protein target for oxathiapiprolin (*Pa*ORP1) and that, based on the high level of
263 sequence identity to its orthologs, it was likely to be highly sensitive to this oomycide.
264
265 *Inhibitory effects on mycelia and zoospores*
266
267 Oxathiapiprolin is best known for its severely inhibitory effects on mycelial growth
268 (Gray et al. 2018; Ji and Csinos 2015; Miao et al. 2016b; Miao et al. 2016a). Its effects
269 on mycelial growth of the two *P. agathidicida* isolates (3770 and 3772) were tested at
270 concentrations ranging from 0.01 ng/ml to 4 ng/ml. For both isolates the EC_{50} was
271 calculated to be approximately 0.1 ng/ml (Table 1). This indicates that *P. agathidicida*
272 mycelia are approximately 5- to 10-fold more sensitive to oxathiapiprolin than those from
273 well-characterized species such as *P. capsici* and *P. nicotianae* (Table 1), with a
274 similarly low EC_{50} to that determined for *P. syringae* (Gray et al. 2018).
275 The motile zoospores of *P. agathidicida* are a potential target for antimicrobials
276 because it is they that initiate new infections by encysting and germinating on the root of
277 the host kauri (Bellgard et al. 2016). We previously tested a range of antimicrobials and
278 plant-derived natural products for their effects on the germination and motility of *P.*
279 *agathidicida* zoospores (Lawrence et al. 2017; Lawrence et al. 2019). Oxathiapiprolin
280 inhibits zoospore germination and (more weakly) motility in other oomycetes (Cohen
281 2015; Gray et al. 2018; Ji and Csinos 2015). Therefore, we set out to implement our
282 previous methods to determine the effects of oxathiapiprolin on *P. agathidicida*
283 zoospores.
284 The EC_{50} values for inhibiting zoospore germination in isolates 3770 and 3772
285 were 9 ng/ml and 2 ng/ml oxathiapiprolin respectively (Table 1). These values are
286 similar to the EC_{50} reported for oxathiapiprolin acting against *P. citrophora* (Gray et al.
287 2018) and from a study on one isolate of *P. capsici* (Miao et al. 2016a), but
288 approximately two orders of magnitude lower than the average determined for 126 *P.*
289 *capsici* isolates in a different study (Ji and Csinos 2015).

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290 For zoospore motility, the results were similar for isolates 3770 and 3772 across
291 three independent preparations of zoospores (Table 2). In controls lacking
292 oxathiapiprolin, zoospores remained motile for 180-240 min. A slight reduction in motility
293 was first seen at 0.001 μg/ml oxathiapiprolin, which was a 10-fold higher concentration
294 than the EC_{50} for mycelial growth. Higher concentrations of oxathiapiprolin reduced
295 zoospore motility to a greater degree, but even at 1 μg/ml (10,000-fold higher than the
296 mycelial EC_{50}) zoospores remained motile for 60 min (Table 2). Comparable results
297 have been observed for *Pseudoperonospora cubensis*, the oomycete pathogen of
298 cucurbits. At 30 min after treatment with 5 μg/ml oxathiapiprolin, 25% of *P. cubensis*
299 zoospores remained motile (Cohen 2015). On the other hand, we previously showed
300 that copper (II) fungicides (Lawrence et al. 2017) and New Zealand native plant-derived
301 flavonoids (Lawrence et al. 2019) rapidly immobilized *P. agathidicida* zoospores at
302 concentrations that were significantly lower than their EC_{50} values for mycelial growth
303 inhibition.
304
305 *Inhibitory effects on oospores*
306
307 Oospores are key survival propagules in soil for many *Phytophthora* species
308 (Erwin and Ribeiro 1996), particularly those such as *P. agathidicida* that do not produce
309 chlamydospores (Weir et al. 2015). They can lie dormant for years and germinate under
310 the right conditions, leading to the formation of sporangia, release of zoospores, and
311 thus new infections. The dormant oospores are easy to transfer between sites, such as
312 on shoes bearing contaminated soil, making them an important consideration for
313 minimizing disease spread. Oxathiapiprolin is highly effective at preventing mycelial
314 mats of *P. nicotianae* from producing oospores (Gray et al. 2018). However, it is
315 technically challenging to produce pure preparations of oospores and therefore, to test
316 the effects of oomycides upon them. To the best of our knowledge, no-one has
317 investigated the direct effects of oxathiapiprolin on oospores themselves. Here, we have
318 used our recently developed protocols for producing purified *P. agathidicida* oospores,

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319 assessing viability, and for triggering their germination *in vitro*, to test the effects of
320 oxathiapiprolin on this key lifecycle stage.
321 First, we assessed the effect of oxathiapiprolin on oospore germination.
322 Oxathiapiprolin concentrations from 0.1 μg/ml to 10 μg/ml completely inhibited
323 germination (Fig. 2A). On the other hand, live/dead staining showed that oxathiapiprolin
324 did not render oospores non-viable when applied at the same concentrations (Fig. 2B).
325 That is, *P. agathidicida* oospores can withstand oxathiapiprolin treatment but cannot
326 successfully germinate in its presence. Oospores of both *P. agathidicida* isolates (3770
327 and 3772) behaved identically in these tests.
328
329 *Detached Leaf Assays*
330
331 Detached leaf assays provide a simple measure of infectivity on host plants (Goth
332 and Keane 1997; Pettitt et al. 2011). They can be used to assess whether test
333 compounds are preventative and/or curative (Cohen et al. 2018). Here, we tested
334 oxathiapiprolin for its preventative effects. Kauri leaves were damaged and then sprayed
335 with oxathiapiprolin at different concentrations, before being inoculated with each isolate
336 of *P. agathidicida.* The onset of infection for untreated kauri leaves was observed at 4-5
337 days for both isolates. In contrast, pre-treatment with low concentrations of
338 oxathiapiprolin (0.1 μg/ml and 1 μg/ml) delayed the onset of infection until day 8 and day
339 9, respectively (Fig. 3). The highest concentration of oxathiapiprolin (10 μg/ml)
340 completely inhibited infection during the ten-day observation period.
341
342 **Discussion**
343
344 Oxathiapiprolin is a novel oomycide that is highly active against many species of
345 *Phytophthora* at extremely low concentrations (Belisle et al. 2019; Gray et al. 2018; Ji
346 and Csinos 2015; Pasteris et al. 2016). In this study we sought to determine its
347 effectiveness against multiple lifecycle stages of *P. agathidicida*, the causative agent of
348 kauri dieback disease. Similar to observations for other *Phytophthora* species,

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349 oxathiapiprolin was effective against mycelial growth and zoospore germination at
350 concentrations far below 1 μg/ml (Table 1). Indeed, the measured EC_{50} values for
351 mycelial growth indicate that *P. agathidicida* mycelia are even more sensitive to
352 oxathiapiprolin than well-studied species such as *P. capsici* and *P. cinnamomi* (Belisle
353 et al. 2019; Ji and Csinos 2015; Miao et al. 2016b). Oxathiapiprolin was also effective at
354 delaying or preventing the infection of kauri leaves, albeit at higher concentrations (Fig.
355 3). On the other hand, it failed to render zoospores immotile in less than an hour (Table
356 2). Overall, its inhibitory effects were comparable for the two geographically distinct *P.*
357 *agathidicida* isolates 3770 and 3772.

358 In assessing the effects of oxathiapiprolin on the *P. agathidicida* lifecycle, a
359 particular focus was how it affects oospores. This is an often-overlooked aspect of
360 oomycide efficacy. If a compound is effective at inhibiting mycelial growth *in planta*, yet
361 ineffective at inhibiting oospore germination and/or viability, disease can readily re-
362 emerge from tissues containing oospores, or new infections can rapidly initiate from
363 oospore-containing soil. The only previous study of *P. agathidicida* oospores showed
364 that viability is largely unaffected by Trigene (a commercial disinfectant containing
365 halogenated tertiary amines), salt water immersion or various pH treatments, with
366 substantial heat treatment being the only effective measure for reducing viability *in vitro*
367 (Dick and Kimberly 2013). Here we found oxathiapiprolin to be highly effective at
368 inhibiting germination, but like these other treatments it was ineffective at reducing
369 oospore viability (Fig. 2). Our results highlight the importance of assessing as many
370 lifecycle stages as possible when testing new oomycides, in order to obtain a complete
371 picture of their effectiveness.

372 Trunk injection of phosphite is the only chemical treatment currently being used
373 against confirmed cases of kauri dieback (Bradshaw et al. 2020; Horner et al. 2015).
374 Here we have conducted the first tests to establish whether oxathiapiprolin should also
375 be considered. Currently in New Zealand, oxathiapiprolin (sold as Zorvec Enicade:
376 https://www.corteva.co.nz/products-and-solutions/crop-protection/zorvec-enicade.html)
377 is only approved for the control of *Peronospora destructor* (downy mildew) in bulb onion.
378 However, our results show that oxathiapiprolin is also highly effective against several

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379 key lifecycle stages of *P. agathidicida*. By acting at sub-microgram per millilitre
380 concentrations to inhibit mycelial growth, zoospore germination and oospore
381 germination (albeit *in vitro*), oxathiapiprolin emerges as a promising candidate for further
382 testing. Inhibiting zoospore and oospore germination highlights its potential as a
383 preventative agent, whereas its extreme effectiveness against mycelial growth suggests
384 it may also act curatively, even in large trees. Future studies should focus on
385 determining the optimal dosage, application method, uptake and overall efficacy *in*
386 *planta.* Ultimately, field trials of oxathiapiprolin against confirmed cases of kauri dieback
387 will be a critical part of the ongoing efforts to protect kauri, which are unique taonga
388 (treasures) of New Zealand.

389

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391 Biological Sciences at Victoria University of Wellington.

392

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526 **Tables**

527

528

529 **Table 1. EC_{50} values for oxathiapiprolin inhibiting selected life cycle stages of *P.*
530 *agathidicida* and related species.** 95% confidence intervals are listed in parentheses
531 (n = 3 biological replicates).

532

Pathogen	Mycelial growth EC_{50} (ng/ml)	Zoospore germination EC_{50} (ng/ml)
P. agathidicida 3770	0.13 (0.11 – 0.14)	8.5 (5.2 – 13)
P. agathidicida 3772	0.11 (0.096 – 0.11)	1.8 (0.25 – 3.6)
P. capsici[a]	0.68 (0.54 – 0.85)	6.0 (0.64 – 56)
P. capsici[b]	1.0	540
P. citrophthora[c]	0.3	8
P. nicotianae[c]	0.5	NR[d]
P. syringae[c]	0.1	NR[d]

533 [a] As reported by others (Miao et al. 2016a).
534 [b] Average across 126 isolates, as reported by others (Ji and Csinos 2015).
535 [c] Average across multiple isolates, as reported by others (Gray et al. 2018).
536 [d] NR, not reported.

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537

538

539 **Table 2. Oxathiapiprolin inhibition of zoospore motility**. The mean time until
540 complete loss of motility of all zoospores is listed for each oxathiapiprolin concentration
541 (*n* = 3 biological replicates).

542

[Oxathiapiprolin], *μ*g/ml[a]	Isolate 3770	Isolate 3772
0	180 min	240 min
0.001	150 min	210 min
0.01	90 min	120 min
0.02	90 min	90 min
0.04	90 min	90 min
0.08	60 min	90 min
0.16	60 min	60 min
1.0	60 min	60 min

543 [a] Oxathiapiprolin was dissolved in DMSO; therefore DMSO was added to all wells at a final concentration
544 of 1% (v/v) to control for any solvent effects.

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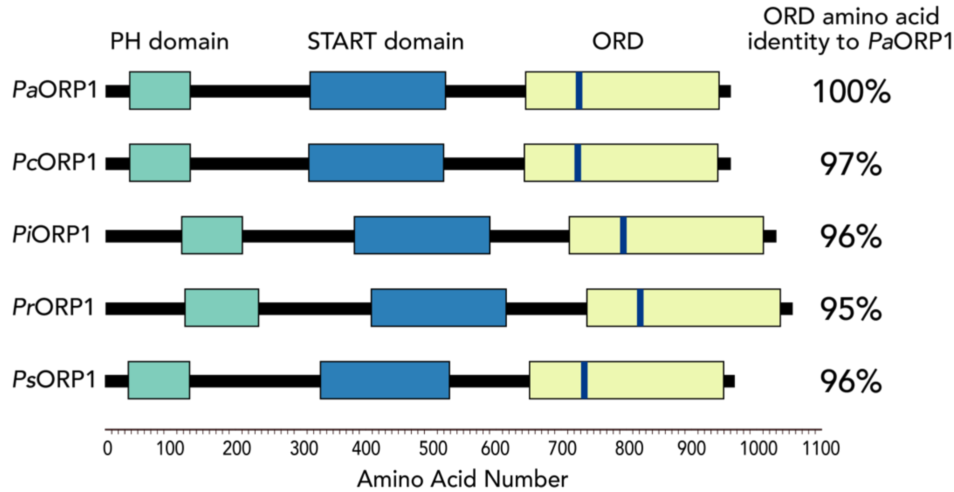
545 **Figures**

546

547



548

549

550 **Fig. 1. Predicted domain architecture of the *Pa*ORP1 protein and comparison with**
551 **related *Phytophthora* species.** *Pa*ORP1 is predicted to be a 960 amino acid protein
552 with an N-terminal pleckstrin homology (PH) domain (green), a StAR-related lipid
553 transfer (START) domain (blue), and the canonical C-terminal OSBP-related domain
554 (ORD; yellow). Within the ORD is the signature motif, EHTSHHPP, indicated by a blue
555 bar. The protein domain architecture is compared with the ORP1 proteins from *P.*
556 *capsici* (*Pc*ORP1), *P. infestans* (*Pi*ORP1), *P. ramorum* (*Pr*ORP1), and *P. sojae*
557 (*Ps*ORP1). The amino acid identity between each ORD and the *P. agathidicida*
558 sequence is also shown.

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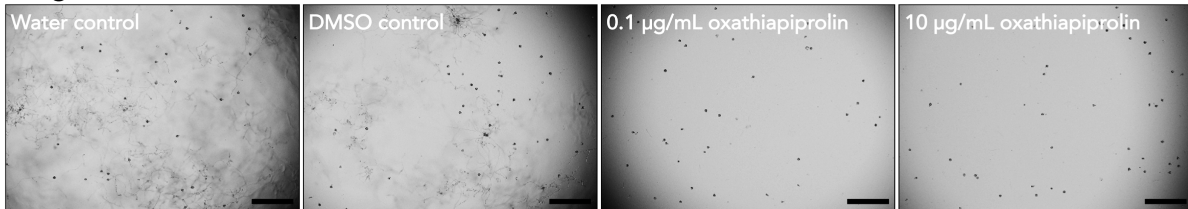
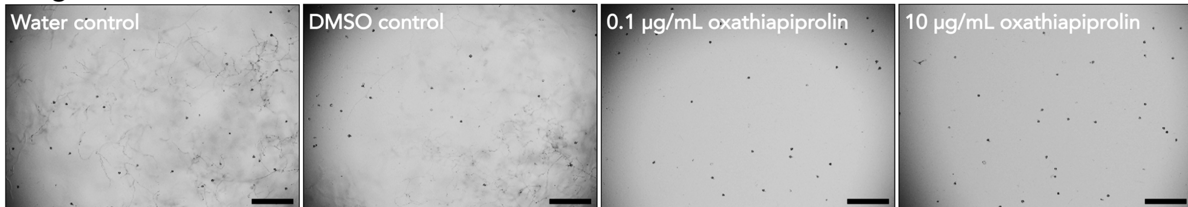
(a)

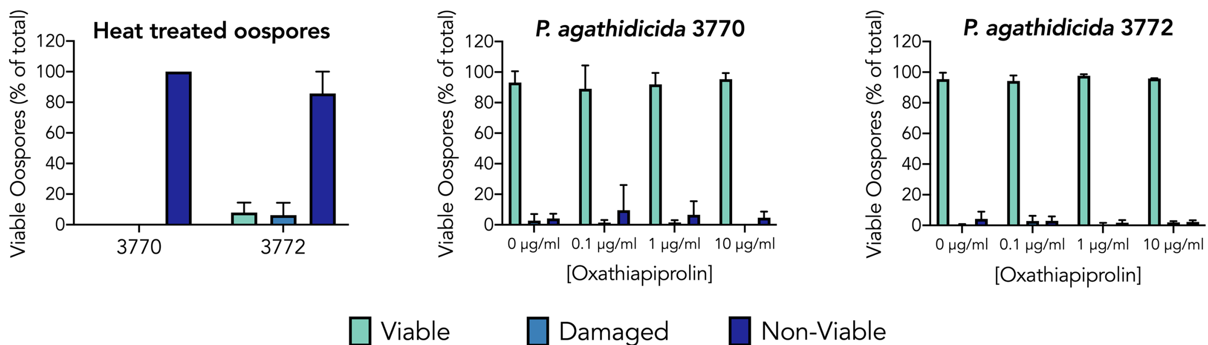
P. agathidicida 3770



P. agathidicida 3772



(b)



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560

561 **Fig. 2. Effects of oxathiapiprolin on *P. agathidicida* oospores.** (**a**) Inverted light
562 microscopy images of oospores triggered to germinate by the addition of kauri root
563 extract, in the presence or absence of oxathiapiprolin. New mycelial mats from
564 germinated oospores could not be captured in a single focal plane so they appear as a
565 combination of fibrils and hazy shadows in the water-only and DMSO controls. Scale bar
566 = 250 *µ*m. (**b**) Results of live/dead staining for oospore viability. The left panel shows the
567 results from heat treating oospores of each *P. agathidicida* isolate, to verify the viability
568 screening method. The other panels show the effects of treating each isolate with
569 varying concentrations oxathiapiprolin. Error bars show the standard deviation from *n* =
570 3 independent oospore preparations.

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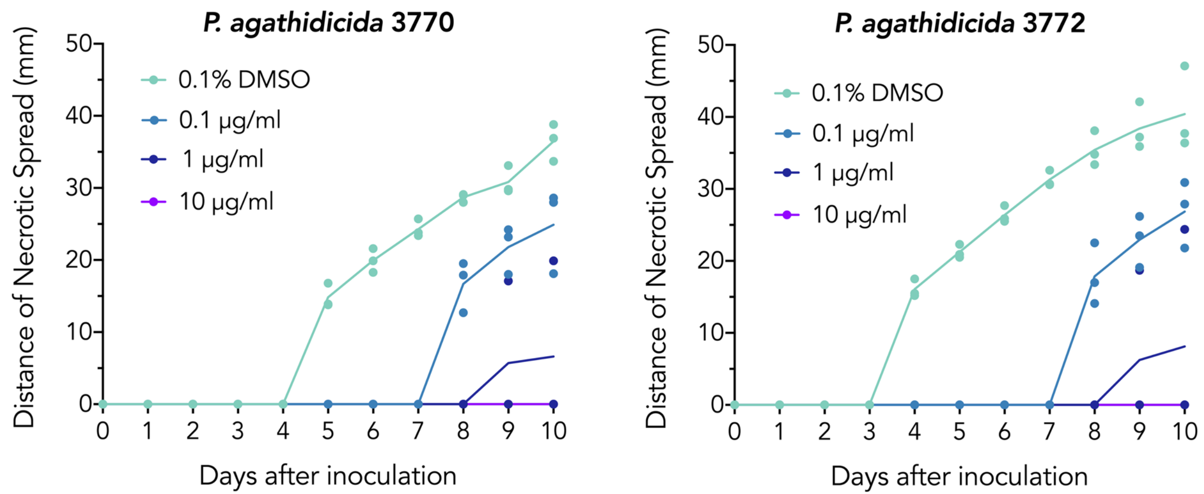

573
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575 **Fig. 3. Preventative effects of oxathiapiprolin on infection of kauri leaves**.
576 Detached leaf assay results for *P. agathidicida* isolates 3770 (left panel) and 3772 (right
577 panel). The oxathiapiprolin concentrations used to pre-treat leaves are indicated and
578 each data point is shown for $n = 3$ independent replicates at each concentration. The
579 connecting lines indicate means of the necrotic spread observed for each treatment.
580 Leaf necrosis indicates *P. agathidicida* infection; therefore, reduced or absent necrosis
581 indicates that the treatment has a preventative effect.