Research Article

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Sensitivity of *Pythium* spp. and *Phytopythium* spp. and tolerance mechanism of *Pythium* spp. to oxathiapiprolin

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Abstract

BACKGROUND: Oxathiapiprolin, developed by DuPont, is the only commercial oxysterol-binding protein inhibitor (OSBPI) of oomycete pathogens. Although the activity of oxathiapiprolin on some *Pythium* spp. and *Phytopythium* spp. has been reported, it has not been tested on many other species, and little is known about the mechanisms of *Pythium* spp. that are tolerant to it.

RESULTS: Oxathiapiprolin exhibited a strong inhibitory effect on mycelial growth of *Phy. litorale*, *Phy. helicoides* and *Phy. chamaehyphon*, with EC_{50} values ranging from 0.002 to 0.013 μg mL^{-1}. It also showed good effectiveness against *Py. splendens* and two *Py. ultimum* isolates, with EC_{50} values ranging from 0.167 to 0.706 μg mL^{-1}, but showed no activity against 14 other *Pythium* spp. Oxathiapiprolin provoked a slight upregulation of *PuORP1* in *Py. ultimum*, but it did not lead to *PaORP1-1* or *PaORP1-2* overexpression in *Py. aphanidermatum*. Transformation and expression of *PuORP1*, *PaORP1-1* or *PaORP1-2* in the sensitive wild-type *Phytophthora sojae* isolate P6497 confirmed that either the *PuORP1*, *PaORP1-1* or *PaORP1-2* was responsible for the observed oxathiapiprolin tolerance.

CONCLUSION: This study showed that oxathiapiprolin had excellent activity against *Phytopythium* spp. but displayed a differentiated activity against different *Pythium* spp. ORP1s in *Pythium* spp. are positively related to the tolerance of *Pythium* species to oxathiapiprolin.

Supporting information may be found in the online version of this article.

1 INTRODUCTION

The genus *Pythium*, with 327 described species (www.mycobank.org), belongs to the family Pythiaceae, order Pythiales, class Oomycetes, phylum Oomycota and kingdom Chromista.[1] *Pythium* species are widely distributed throughout the world, ranging from tropical to temperate and even Arctic and Antarctic regions.[2] They exist as saprophytes, parasites or pathogens in soil and water and on plants, fungi, insects, fish, marine red algae, animals and human beings.[3] Economically, many *Pythium* species are especially important plant pathogenic oomycetes, causing serious damage to agricultural crops and turf grasses, soft rot of fruit, roots and stems, and pre- and post-emergence disease of seeds and seedlings by infecting mainly juvenile tissues.[4]

Although traditional means of disease control are used, including the application of cultural practices such as crop rotation, deep tillage, water management and balanced nutrition, control of *Pythium* spp. infections is mainly accomplished by treatments with limited fungicides such as phenylamides, quinone outside inhibitors (QoIs), ethaboxam, propamocarb and hymexazole.[5–9] However, many pathogens have developed resistance against these fungicides. For example, metalaxyl was registered for use in the U.S. in 1980, and within 4 years, fungicide resistance in *Pythium* causing turf blight was detected.[10] Insensitivity toward ethaboxam or QoIs by *Py. acrogynum*, *Py. recalcitrans* and *Py. aphanidermatum* has been recently reported in Minnesota in the U.S.[11] *Pythium* isolates from commercial greenhouses in Pennsylvania showed dual resistance to propamocarb and mefenoxam.[8] The growing number of field populations with reduced sensitivity toward commercial fungicides has led to increased interest in identifying and evaluating new active ingredients to manage *Pythium* pathogens that affect field crops.

The genus *Phytopythium* is morphologically intermediate between the genera *Phytophthora* and *Pythium* and was formally identified in June 2010.[12, 13] There are 29 associated records for this genus in Mycobank (www.mycobank.org). Although not all species of *Phytopythium* are plant pathogens,[14] some species are highly plant pathogenic. For example, *Phy. helicoides* had been reported to cause rhizome rot of Asian lotus, which is an economically important aquatic plant in China. This pathogen also causes stem rot of Shatangju mandarin seedlings.[15, 16] To the best of our knowledge, there is no registered fungicide to control *Phytopythium* diseases in China.

Oxathiapiprolin is the first member of a new class of piperidinyl thiazole isoxazoline fungicides to be developed by DuPont.[17] It had been confirmed that the molecular target of oxathiapiprolin

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is the oxysterol-binding protein (OSBP),[17] which is a member of the OSBP-related proteins (ORPs) family. Since the function of ORPs in oomycetes remains unknown, we named the target protein of oxathiapiprolin as ORP1, such as PcORP1 (Genome protein ID: 564296) in *Phytophthora capsici* and PiORP1 (GenBank accession number: XP_002902250.1) in *Ph. infestans*.[18, 19] Oxathiapiprolin showed excellent activity on downy mildew pathogens and *Phytophthora* spp. with EC_{50} values of inhibition mycelial growth at about 10^{-4} μg mL^{-1}.[20] Vargas (2018) reported that oxathiapiprolin significantly limited the mycelial growth of *Phy. helicoides*, *Phy. litorale*, *Phy. delawarense* and *Phy. mercuriale*, with EC_{50} values ranging from 0.00043 to 0.01803 μg mL^{-1}, but has no effect on the mycelial growth of 12 *Pythium* isolates (four *Py. ultimum* isolates, two *Py. lutarium* isolates, two *Py. attrantheridium* isolates and one *Py. torulosum* isolate), at 0.1 μg mL^{-1}.[21] However, according to our previous results, oxathiapiprolin showed good activity against *Py. ultimum* with EC_{50} values of 0.085 μg mL^{-1}.[20] Thus, *Py. ultimum* isolates with diffent sources might display different sensitivity to oxathiapiprolin.

Therefore, the objectives of this study were to (i) further evaluate the activity of oxathiapiprolin to a wider range of *Pythium* spp. and *Phytopythium* spp. and (ii) analyze the mechanism of different sensitivities of *Pythium* spp. to oxathiapiprolin.

2 MATERIALS AND METHODS

2.1 Fungicides

Oxathiapiprolin (96.7%, technical grade), provided by DuPont Crop Protection (Wilmington, DE), was dissolved in dimethyl sulfoxide (DMSO) to produce a stock solution with a 10^4 μg a.i. mL^{-1}, which was then stored in the dark at 4 °C until required.

2.2 Sensitivity of Pythium spp., Phytopythium spp. and Phytophthora sojae

Phy. litorale, *Phy. helicoides* and *Phy. chamaehyphon*, as well as *Py. ultimum* ZJFM 2, *Py. splendens*, *Py. arrhenomanes*, *Py. carollinianum*, *Py. dissotocum*, *Py. delicense*, *Py. heterothallicum*, *Py. hydnosporum*, *Py. intermedium*, *Py. irregulare*, *Py. myriotylum*, *Py. sylvaticum*, *Py. spinosum* and *Py. oligandrum* were provided by Professor Daolong Dou (Nanjing Agricultural University of China). *Py. guiyangense* was provided by Professor Xiaoqing Su (Guizhou Medical University). *Py. aphanidermatum* and *Py. ultimum* NWAFU-1 were obtained from infected cucumber samples collected from the field (Table 1). All *Pythium* spp. and *Phytopythium* spp. (Table 1) were cultured and maintained on potato dextrose agar (PDA) medium. The efficacy of oxathiapiprolin on the mycelial growth of 17 *Pythium* spp. and three *Phytopythium* spp. on PDA was investigated according to the protocol of our previous study.[20] At first, one initial discriminatory concentration (50 μg mL^{-1}) was used. If oxathiapiprolin (50 μg mL^{-1}) showed excellent inhibitory activity to some strains, various concentrations were designed (Table S1), and the median effective concentration (EC_{50}) was calculated using a linear model regressing the relative growth values (as a proportion of the control) against the log-transformed oxathiapiprolin concentrations. The final concentration of DMSO in the medium was 0.1% (v/v) in all treatments. Each treatment consisted of three replicate plates. The diameter of each colony was measured perpendicularly after 2 days of incubation at 25 °C. The experiment was performed three times.

The sensitivity of *Ph. sojae* isolates to oxathiapiprolin was also determined using the mycelia growth assay described in our previous study.[20] Mycelial plugs (5 mm) were taken from the colony margin and transferred to fresh 10% V8 agar amended with various concentrations (0.005, 0.45, 10 μg mL^{-1}) of oxathiapiprolin. The final concentration of DMSO in the medium was 0.1%, and plates containing only 0.1% DMSO were used as the negative control. The diameters of the oxathiapiprolin cultures were measured after 3 days' growth. All the plates were incubated in darkness at 25 °C. Each experiment contained three replica plates and the experiment was repeated three times.

2.3 Bioinformatics

To identify the *ORP1* genes in different species, the NCBI (https://blast.ncbi.nlm.nih.gov) and FungiDB (https://fungidb.org/fungidb) databases were searched by BlastP using PiORP1 (GenBank accession number: XP_002902250.1) as a query. Conserved domains were analyzed using CD-search in NCBI (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). Multiple alignments were generated using DNAMAN 6.0.

2.4 Nucleic acid isolation from *Pythium* spp. and *Ph. sojae*

RNA used to investigate the expression levels of the *PaORP1-1* and *PaORP1-2* in *Py. aphanidermatum* and *PuORP1-1* in *Py. ultimum* NEAFU-1 was prepared by inoculating 60 mL potato dextrose broth (PDB) media with five mycelial plugs of either species (5 mm in diameter). The flasks were then incubated at 25 °C for 12 h in a rotary shaker at 120 rpm before oxathiapiprolin (0, 0.2 or 0.4 μg mL^{-1}) was added to the treatment flasks. The mycelia were harvested by vacuum filtration after a further 24 h incubation and frozen at −80 °C until required. For transformants verification, mycelia were harvested from *Ph. sojae* isolates grown in 10% V8 liquid medium[22] for 3 days and frozen at −80 °C until required. Total RNA was extracted from the frozen samples using the SV Total RNA Isolation kit (Promega Corp., Madison, WI, USA), and cDNA was synthesized using the FastKing RT kit (with gDNase; Tiangen Biotech, Beijing, China), with each procedure being conducted according to the manufacturer's protocol. Total DNA was isolated using Ristaino *et al.*'s (1998) method.[23]

2.5 Quantitative real-time polymerase chain reaction analysis of transcript levels

The primers used for the quantitative real-time polymerase chain reaction (qRT-PCR) analysis of the *PaORP1-1*, *PaORP1-2* and *PuORP1-1* were listed in Table S2. qRT-PCR analyses were performed using the CFX Connect™ Real-Time system (Bio-Rad, Munich, Germany). Reaction mixtures had a final volume of 20 μL and consisted of 10 μL of 2 × SuperReal Color PreMix (SYBR Green; Tiangen Biotech, Beijing, China), 0.6 μL of each primer (10 μM) and 1 μL of cDNA that had been diluted 4-fold. The PCR was processed using the following program: 95 °C for 15 min, followed by 40 cycles of 10 s at 95 °C, 32 s at 60 °C and 30 s at 72 °C. The relative quantities of the PCR products were calculated using the $2^{-\Delta\Delta Ct}$ method, and the *PaActin* gene (GenBank accession number: JN038402)[24] or *PuActin* gene (Transcript ID: PYU1_T009609) was used as a reference to normalize the quantification of the *PaORP1-1*, *PaORP1-2* and *PuORP1-1* transcript levels, respectively. The entire experiment was conducted twice, and each experiment included three replicates for each treatment.

2.6 Transformation of *Ph. sojae*

The complete coding sequences of *PaORP1-1*, *PaORP1-2* and *PuORP1-1* were amplified from the cDNA of *Py. aphanidermatum* and *Py. ultimum* NWAFU-1, respectively, using the primers list in

Table 1 Lists of isolates of *Phytopythium* spp. and *Pythium* spp. used in this study

Isolate	Isolate code	Year collected	Province collected	Source
Phytopythium litorale	CAU-11	2014	Beijing	water
Phytopythium helicoides	NWAFU-7	2017	Shangdong	lotus
Phytopythium chamaehyphon	NWAFU-11	2015	Beijing	water
Pythium ultimum	NWAFU-1	2015	Beijing	cucumber seedling
Pythium ultimum	ZJFM 2	2016	Shandong	strawberry
Pythium splendens	Chen 43	2016	Hunan	soil
Pythium arrhenomanes	Par 3	2016	Henan	soil
Pythium aphanidermatum	NWAFU-10	2015	Beijing	cucumber seedling
Pythium carollinianum	NWAFU-14	2016	Shandong	soil
Pythium dissotocum	Chen 79	2015	Hunan	soil
Pythium deliense	NWAFU-12	2015	Beijing	soil
Pythium heterothallicum	Chen 44	2016	Hunan	soil
Pythium hydnosporum	ACCC-36841	2016	Beijing	soil
Pythium intermedium	Chen 60	2016	Hunan	soil
Pythium irregulare	NWAFU-13	2016	Beijing	soil
Pythium myriotylum	Chen 134	2016	Jiangsu	soil
Pythium sylvaticum	NWAFU-17	2016	Shandong	garlic
Pythium spinosum	Chen 82	2015	Hunan	soil
Pythium oligandrum	CAU-13	2014	Shandong	soil
Pythium guiyangense	NWAFU-18	2018	Guizhou	Asian tiger mosquito

Table S2. The 50 μL PCR reaction mixes contained 1 × PCR buffer, 100 ng of DNA, 0.2 μM each primer, 0.2 mM each dNTP, and 2.5 U of *TransStart® FastPfu* DNA polymerase (TransGen Biotech Co., Beijing, China) and processed in a T100™ Thermal Cycler (Bio-Rad, Singapore) with the following program: 2 min at 95 °C, 35 cycles of 20 s at 95 °C, 20 s at 60 °C, and 1 min at 72 °C, followed by a final extension step of 5 min at 72 °C. The PCR products were sequenced by Shanghai Sangon Biotech Co. Ltd. Then the fragments were ligated into the PYF3 expression vector as *Apa* I/*Sac* II fragments. The sequences of the insertions were verified by DNA sequencing. Then, the corresponding plasmid was transformed into the oxathiapiprolin-sensitive wild-type *Ph. sojae* isolate P6497 by PEG/$CaCl_2$-mediated protoplast transformation.[25]

The transformants produced were screened by PCR using genomic DNA (100 ng) and cDNA (1 μL) as a template with the primers shown in Table S2 to confirm the presence and expression of the transgenes, respectively. The PCR was performed as described above but with a shorter extension period of 15 s. After the PCR was completed, a 5 μL aliquot of the PCR product from each sample was analyzed by electrophoresis using a 1.5% agarose gel.

3 RESULTS

3.1 *In vitro* activity of oxathiapiprolin against *Phytopythium* spp. and *Pythium* spp.

Oxathiapiprolin exhibited a strong inhibitory effect on mycelial growth of *Phy. litorale*, *Phy. helicoides* and *Phy. chamaehyphon*, with EC_{50} values of 0.013, 0.002 and 0.002 μg mL^{-1} and EC_{90} values of 0.047, 0.005 and 0.007 μg mL^{-1}, respectively (Table 2). However, different *Pythium* species showed different fungicide sensitivities, and oxathiapiprolin was only effective against *Py. splendens* and two *Py. ultimum* isolates, with EC_{50} values ranging from 0.167 to 0.706 μg mL^{-1} and EC_{90} values ranging from 0.375 to 3.039 μg mL^{-1}, respectively (Table 2). Oxathiapiprolin did not limit the growth of the other 14 *Pythium* species (Table 2).

3.2 Analysis of PiORP1 homologous proteins in *Phytopythium* spp. and *Pythium* spp.

The full-length sequences of PiORP1 homologous proteins in *Phy. vexans* and eight *Pythium* species were identified. One PiORP1 homologous protein (long ORPs) was found in *Phy. vexans* (Transcript ID: EPrPVT00000016714), *Py. ultimum* (Transcript ID: PYU1_T006745), *Py. arrhenomanes* (Transcript ID: EPrPRT00000016228), *Py. irregulare* (Transcript ID: EPrPIT00000023012) and *Py. iwayamai* (Transcript ID: PIW_T008063-RA), and two homologous ORP1 (named ORP1-1 and ORP1-2, respectively) were obtained in *Py. aphanidermatum* (Transcript ID: EPrPAT00000022195 and EPrPAT00000016161), *Py. guiyangense* (Protein ID: PG3S_12589 and PG3S_18892), *Py. oligandrum* (GenBank accession number: TMW64014.1 and TMW56818.1) and *Py. insidiosum* (GenBank accession number: GAY05260.1 and GAX98766.1). OSBP-related domains (ORDs) of ORP1s from different oomycetes, which are the binding domain of oxathiapiprolin,[26] were analyzed and aligned (Fig. 1(a)). The ORD of PiORP1 had 72.9% homology with that of PvORP1 from *Phy. vexans* and less than 70% homology with those from other *Pythium* species (Fig. 1(b)).

3.3 Back-transformation of PuORP1, PaORP1-1, and PaORP1-2 in Phytophthora sojae

Vectors containing the full-length *PuORP1*, *PaORP1-1* or *PaORP1-2* were used to transform the oxathiapiprolin-sensitive wild-type *Ph. sojae* isolate P6497. Seven independent transformants were recovered: two containing *PuORP1* (TU1-5 and TU1-6), two containing *PaORP1-1* (TA1-2 and TA1-4), two containing *PaORP1-2* (TA2-5 and TA2-6) and one containing the empty vector (TCK-1). RT-PCR results showed that the transferred genes were expressed in the transformants (Fig. 2(a)). With the exception of TCK-1 and wild-type P6497, all the other transformants could grow on 10% V8 agar amended with oxathiapiprolin up to 10 μg mL^{-1} (Fig. 2 (b) and (c)).

Table 2 Toxicity of oxathiapiprolin to mycelial growth of three *Phytopythium* spp. and 16 *Pythium* spp.

Isolate	EC_{50} (μg mL^{-1})	EC_{90} (μg mL^{-1})	Regression equation
Phytopythium litorale	0.013 (0.012–0.015)[a]	0.047 (0.039–0.058)	Y = 4.407 + 2.348X
Phytopythium helicoides	0.002 (0.001–0.002)	0.005 (0.003–0.014)	Y = 7.673 + 2.777X
Phytopythium chamaehyphon	0.002 (0.002–0.003)	0.007 (0.006–0.008)	Y = 7.265 + 2.786X
Pythium ultimum 1	0.167 (0.154–0.180)	0.375 (0.336–0.434)	Y = 2.840 + 3.658X
Pythium ultimum 2	0.401 (0.345–0.463)	2.049 (1.620–2.775)	Y = 0.712 + 1.796X
Pythium splendens	0.706 (0.617–0.797)	3.039 (2.523–3.845)	Y = 0.306 + 2.021X
Pythium arrhenomanes	>50	>50	-
Pythium aphanidermatum	>50	>50	-
Pythium carollinianum	>50	>50	-
Pythium dissotocum	>50	>50	-
Pythium deliense	>50	>50	-
Pythium heterothallicum	>50	>50	-
Pythium hydnosporum	>50	>50	-
Pythium intermedium	>50	>50	-
Pythium irregulare	>50	>50	-
Pythium myriotylum	>50	>50	-
Pythium sylvaticum	>10	>10	-
Pythium spinosum	>50	>50	-
Pythium oligandrum	>50	>50	-
Pythium guiyangense	>50	>50	-

[a]Values in parentheses are 95% confidence limits.

3.4 Expression levels of *PuORP1*, *PaORP1-1*, *PaORP1-2* in *Py. ultimum* and *Py. aphanidermatum* in the presence or absence of oxathiapiprolin

In *Py. aphanidermatum*, oxathiapiprolin treatments did not result in target proteins (PaORP1-1 and PaORP1-2) overexpression, but downregulation of the transcript levels of *PaORP1-1* and *PaORP1-2* (Fig. 3). However, oxathiapiprolin at 0.4 μg mL^{-1} provoked a slight upregulation of *PuORP1* in *Py. ultimum* (Fig. 3).

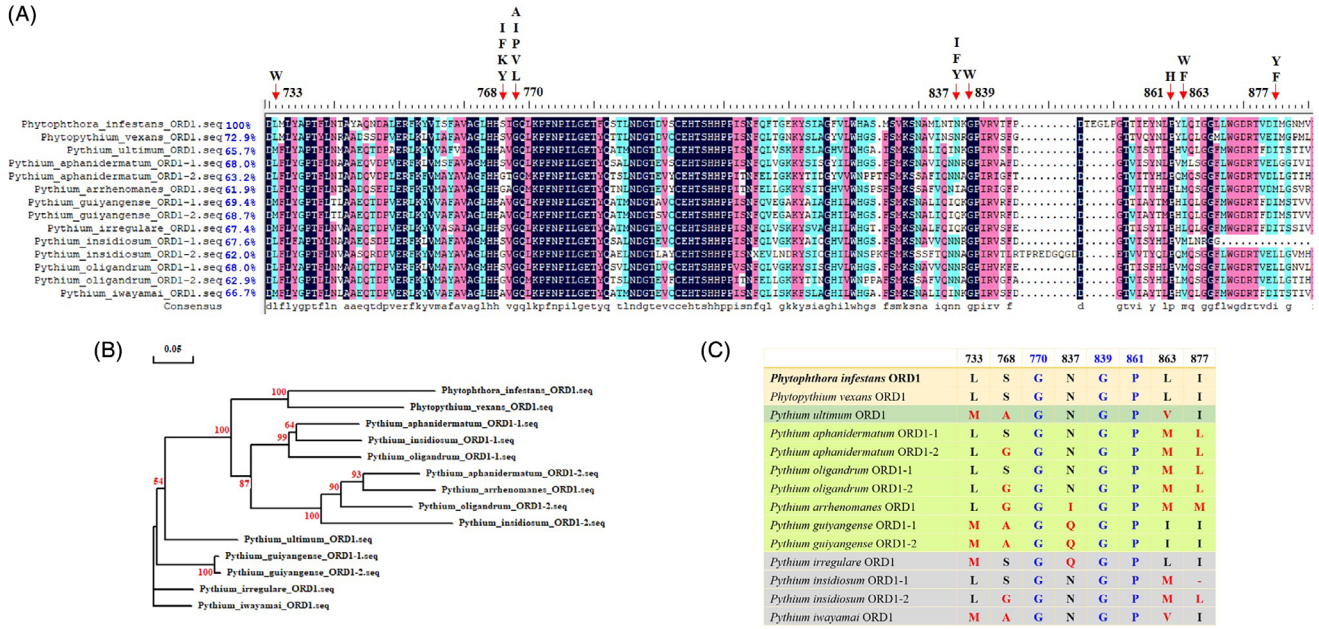
4 DISCUSSION

In this study, oxathiapiprolin showed excellent activity against three *Phytopythium* species *in vitro*. However, compared with the EC_{50} value (6.78×10^{-4} μg mL^{-1}) of oxathiapiprolin to *Ph. capsici*,[20] three tested *Phytopythium* species showed partially tolerant to oxathiapiprolin. Thus, further study is needed to confirm whether oxathiapiprolin could effectively control *Phytopythium* diseases in aquatic environments.

Oxathiapiprolin showed no activity against plant pathogenic *Py. aphanidermatum*, *Py. arrhenomanes*, *Py. dissotocum*, *Py. deliense*, *Py. heterothallicum*, *Py. intermedium*, *Py. irregulare*, *Py. myriotylum*, *Py. sylvaticum* and *Py. spinosum* even at 50 μg mL^{-1}. Furthermore, this fungicide had no effect on *Py. hydnosporum*, which is a potential danger to the commercial cultivation of *Agaricus bisporus*.[27] Importantly, oxathiapiprolin did not inhibit three beneficial *Pythium* species: *Py. oligandrum* (mycoparasite),[28] *Py. guiyangense* (a parasite of mosquito larvae)[29] and *Py. carollinianum* (a mosquito-killing oomycetes).[30] *Py. oligandrum* has been registered in China to control tomato late blight caused by *Ph. infestans*. Thus, rotational and mixed application of oxathiapiprolin and *Py. oligandrum* could be considered in the field and could be used as a fungicide resistance management strategy.

In a previous study, oxathiapiprolin (0.1 μg mL^{-1}) showed no activity against *Py. torulosum*, *Py. ultimum var. ultimum*, *Py. lutarium*, *Py. attrantheridium*, *Py. inflatum*, *Py. oopapillum*, *Py. diclinum*, *Py. pleroticum*, *Py. heterothallicum*, *Py. nodosum*, *Py. middletonii* and *Py. dissotocum*.[21] Interestingly, in this study, oxathiapiprolin showed good inhibitory activity against mycelial growth of *Py. ultimum* (two isolates) and *Py. splendens* with EC_{50} values ranging from 0.167 to 0.706 μg mL^{-1}. Different activities were obtained for *Py. ultimum*, which may be caused by the different genetic backgrounds of the tested *Py. ultimum* isolates. It has been observed in the establishment of baseline sensitivity of a pathogen to a new compound that different isolates with different genetic backgrounds showed diverse fungicide sensitivities.[31–33] Lerksuthirat *et al.* (2017) investigated the sensitivity of 30 *Py. insidiosum* isolates to terbinafine and itraconazole, and there were significant sensitivity differences among these isolates.[34] *Py. splendens* is an important plant pathogen, causing severe wilt of muskmelon, seedling root rot of Micheli and stem rot of Guiana chestnut.[35–37] Oxathiapiprolin may be a potential treatment for diseases caused by *Py. ultimum* and *Py. splendens*. However, EC_{50} values of oxathiapiprolin to the tested *Py. ultimum* isolates and *Py. splendens* were about 200–1050 times higher than to *Ph. capsici*. Thus, more *Py. ultimum* and *Py. splendens* isolates should be tested to confirm whether oxathiapiprolin has activity against these two species *in vitro* and *in vivo*.

Py. ultimum and *Py. aphanidermatum* did not have significantly altered *ORP1* expression in the absence of oxathiapiprolin (0.2 μg mL^{-1}). This result provided strong evidence that the tolerance mechanism of *Pythium* spp. to oxathiapiprolin is unlikely to be high expression of the target protein. Then the sequences of ORD domain (the binding domain of oxathiapiprolin in ORP1) from *Phy. vexans* and eight *Pythium* species were analyzed. According to previous reports, point mutations in eight amino acids (733, 768, 770, 837, 839, 861, 863 and 877)[26] were found in the ORD domain of oxathiapiprolin-insensitive *Phytophthora* spp., implying that these are the most prominent positions



	733	768	770	837	839	861	863	877
***Phytophthora infestans* ORD1**	L	S	G	N	G	P	L	I
Phytopythium vexans ORD1	L	S	G	N	G	P	L	I
Pythium ultimum ORD1	M	A	G	N	G	P	V	I
Pythium aphanidermatum ORD1-1	L	S	G	N	G	P	M	L
Pythium aphanidermatum ORD1-2	L	G	G	N	G	P	M	L
Pythium oligandrum ORD1-1	L	S	G	N	G	P	M	L
Pythium oligandrum ORD1-2	L	G	G	N	G	P	M	L
Pythium arrhenomanes ORD1	L	G	G	I	G	P	M	M
Pythium guiyangense ORD1-1	M	A	G	Q	G	P	I	I
Pythium guiyangense ORD1-2	M	A	G	Q	G	P	I	I
Pythium irregulare ORD1	M	S	G	Q	G	P	L	I
Pythium insidiosum ORD1-1	L	S	G	N	G	P	M	-
Pythium insidiosum ORD1-2	L	G	G	N	G	P	M	L
Pythium iwayamai ORD1	M	A	G	N	G	P	V	I

Figure 1 Sequence analyses of ORD (OSBP-related domain) of ORP1s from 10 oomycetes. (A) Amino acid sequence alignment. The percentages indicate the amino sequence identity of each ORD with the ORD of PiORP1 of *Phytophthora infestans*. Eight mutation sites and amino acid substitutions associated with oxathiapiprolin resistance are indicated with red arrows and lists of the substitutions. Dashes indicate gaps in the alignment, and color highlights indicate residues partially or fully conserved among the different ORDs. (B) Phylogenetic relationships of ORD domains. The phylogram was produced using DNAMAN with a maximum likelihood algorithm and 1000 bootstrap replicates. Bootstrap values are shown if >50. The scale bar indicates the relative length of each branch proportional to the number of amino acid changes. (C) Eight prominent amino acid sites affecting sensitivity to oxathiapiprolin in ORD1s from different oomycetes.

affecting sensitivity to OSBPIs. All of these eight amino acids were the same in ORD domain of PiORP1 (PiORD1) and ORD domain of PvORP1 (PvORD1). For other ORD1s in *Pythium* species, at least two amino acids were different compared with wild-type PiORD1 (Fig. 1(c)). However, for 19 mutations in eight amino acids (L733W, S768I/F/K/Y, G770A/I/P/V/L, N837I/F/Y, G839W, P861H, L863W/F, and I877F/Y) conferring oxathiapiprolin resistance in PcORP1,[26] only I837 was found in *Py. arrhenomanes* ORP1 and all other amino acids after mutation were not found in eight *Pythium* species (Fig. 1). Interestingly, N837I had also been detected in oxathiapiprolin-resistant *Plasmopara viticola* isolates obtained in the filed.[26] Except for these eight amino acids, there were many

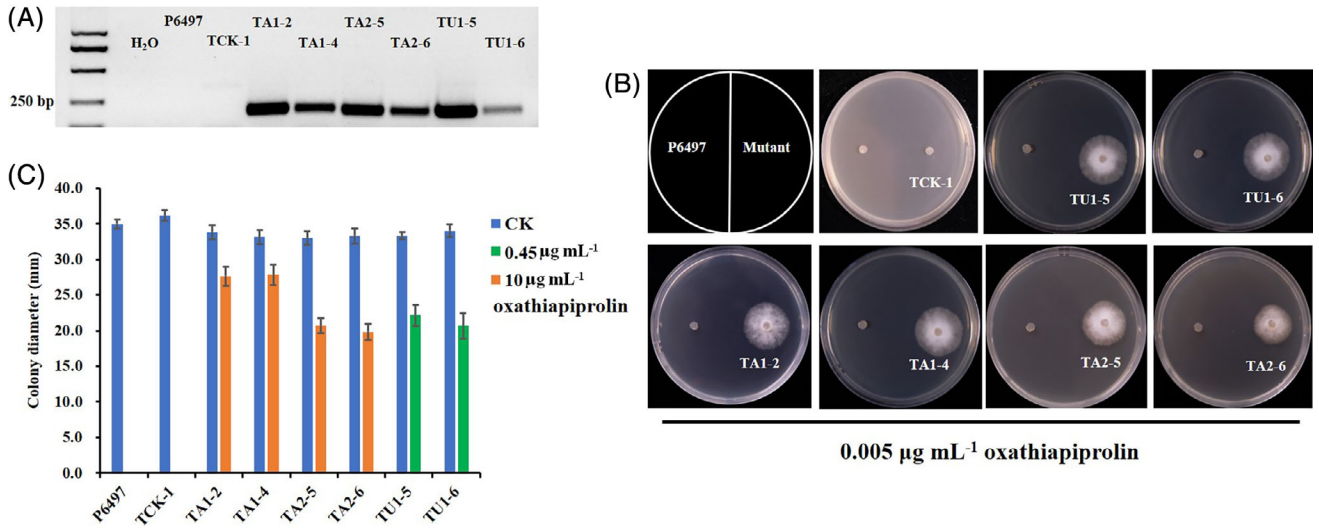


Figure 2 RT-PCR confirmation results of the *Phytophthora sojae* transformants (A) and sensitivity of *Ph. sojae* transformants to oxathiapiprolin (B, C). Transformants were obtained using PEG/$CaCl_2$-mediated protoplast transformation with plasmid containing *PaORP1-1*, *PaORP1-2* and *PuORP1-1*, respectively. P6497 is the sensitive wild-type *Ph. sojae* used for transformation. TCK-1 contains the empty vector (PYF3); TA1-2 and TA1-4 carry the *PaORP1-1* from *Pythium aphanidermatum*; TA2-5 and TA2-6 carry the *PaORP1-2* from *Py. aphanidermatum*; TU1-5 and TU1-6 carry the *PuORP1* from *Py. ultimum*. Mycelial growth was measured after 3 days' growth on 10% V8 agar medium amended with the indicated concentrations of oxathiapiprolin. All the plates were incubated in darkness at 25 °C. Each experiment contained three replica plates and the experiment was repeated three times. Bars indicate standard deviations.

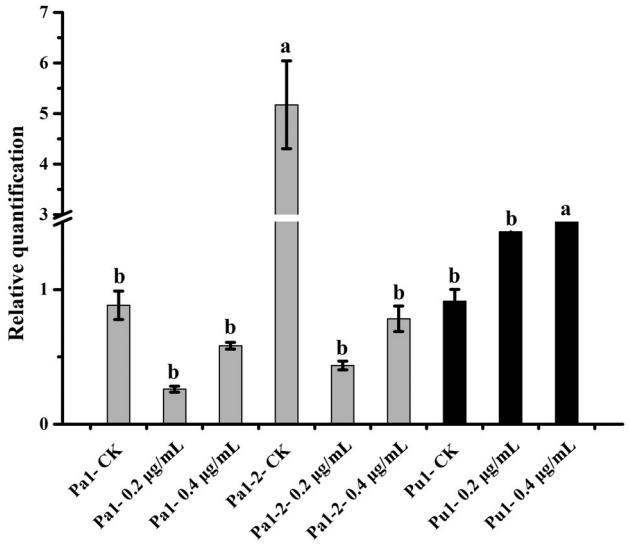


Figure 3 Transcript levels of *PaORP1-1*, *PaORP1-2* in *Pythium aphanidermatum* and *PuORP1* in *Py. ultimum* in the presence or absence of oxathiapiprolin. Transcript levels of three genes were calculated using the $2^{-\Delta\Delta Ct}$ method, and the *PaActin* gene or *PuActin* gene was used as a reference to normalize the quantification. For *PaORP1-1* and *PaORP1-2*, expression levels were calibrated to the *PaORP1-1* in the absence of the fungicide (Pa1-CK, value of 1). For *PuORP1*, the transcript levels were shown relative to the *PuORP1* in the absence of the fungicide (Pu1-CK, value of 1). Columns and bars indicate means ± standard deviation. Columns marked with the same letter are not significantly different by Fisher's protected least significant difference test ($P < 0.05$).

different points between PiORD1 and ORD1s from *Pythium* species. Therefore, we speculate that all the amino acids that were different between PiORD1 from oxathiapiprolin-sensitive *Ph. infestans* and ORD1s from *Pythium* species have led to the oxathiapiprolin tolerance of *Pythium* spp.

To confirm the tolerance mechanism of *Pythium* spp., we attempted to replace the *PsORP1* gene with *ORP1* gene from *Pythium* spp. using the CRISPR-Cas9 system in *Ph. sojae*. Unfortunately, no positive transformants were recovered. Thus, the full-length transcripts of *PuORP1*, *PaORP1-1* and *PaORP1-2* were transformed directly into the sensitive *Ph. sojae* isolate P6497, respectively. All six transformants recovered had reduced sensitivity to oxathiapiprolin. Taken together, these results provide strong evidence that *ORP1s* from *Pythium* spp. were positively related with oxathiapiprolin-tolerance in *Pythium* spp., even though it showed good activity against two tested *Py. ultimum* isolates. The three-dimensional structure of any ORP1 remains unknown. This prohibits predicting the influence of amino acid changes at the key positions on the structural integrity of the protein. Thus, it can only be speculated that the three-dimensional structure of ORP1s from *Pythium* spp. binds weakly with oxathiapiprolin.

Until now, there are a very limited number of alternative chemicals to prevent or treat plant *Pythium* diseases. As far as we know, only mefenoxam, azoxystrobin and hymexazol have been registered in China to control *Pythium* diseases. Oxathiapiprolin showed excellent activity against *Phytophthora* and downy mildews by binding OSBP. Although it showed no activity to most *Pythium* spp., we think structural optimization of oxathiapiprolin may still be a good choice for the development of a new fungicide for *Pythium* spp. because the ORP1s from *Pythium* spp. and *Phytophthora* spp. shared a high homology. But the molecular binding bases of oxathiapiprolin and its target protein need to be clarified at first. Thus, the structure and function of ORP1 protein in *Pythium* spp. needs to be further investigated.

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SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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