Research

Evaluation of New Oomycota Fungicides for Management of Phytophthora Root Rot of Citrus in California

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Abstract

Phytophthora root rot, caused by several species of *Phytophthora*, is an important disease of citrus in California and other growing regions. For chemical management, mefenoxam and potassium phosphite have been available for many years, and resistance in *Phytophthora* spp. has been reported for both compounds. We evaluated the efficacy of the new Oomycota fungicides ethaboxam, fluopicolide, mandipropamid, and oxathiapiprolin, each with a different mode of action, against Phytophthora root rot of citrus in field and greenhouse studies. Root balls of navel orange trees on 'Carrizo citrange' rootstock were inoculated with *P. nicotianae* at planting in the field in fall 2013. Applications with 11 fungicide treatments were made 5 weeks after planting, in spring and fall 2014, and in spring 2015. Feeder roots and adjacent soil were collected before or after application. All of the new fungicides significantly reduced root rot incidence and *Phytophthora* soil populations to very low levels as compared with the control starting after the first

application. Mefenoxam was only effective when a high label rate was used in the fourth application. Selected treatments also increased tree canopy size, trunk diameter, and fruit yield as compared with the control. A rate comparison with the four new fungicides was initiated in summer 2016 in another field trial using navel orange trees inoculated with *P. citrophthora*. Minimum effective rates to reduce Phytophthora root rot incidence and pathogen soil populations were determined after one and two applications in fall 2016 and summer 2017, respectively. Greenhouse studies confirmed the efficacy of the new fungicides. Based in part on our studies, fluopicolide recently received a federal and oxathiapiprolin a full registration for use on citrus, and registrations for ethaboxam and mandipropamid have been requested. These new compounds will provide highly effective treatment options and resistance management strategies using rotation and mixture programs for the control of Phytophthora root rot of citrus.

Phytophthora root rot is one of most destructive diseases of citrus and occurs worldwide (Erwin and Ribeiro 1996). Symptoms begin with a brown discoloration and dieback of feeder roots. If the damage of feeder roots exceeds their regeneration, adequate water and nutrient uptake will be impeded, resulting in a slow decline of the tree. The disease can be caused by several soilborne *Phytophthora* species, including *Phytophthora citrophthora* (R.E. Sm. & E.H. Sm.) Leonian and *P. nicotianae* Breda de Haan (syn. *P. parasitica* Dastur) as well as *P. cactorum* (Lebert & Cohn) J. Schröt., *P. drechsleri* Tucker, *P. megasperma* Drechsler, and *P. palmivora* (E.J. Butler) E.J. Butler (Erwin and Ribeiro 1996). In California, *P. nicotianae* and *P. citrophthora* are the major causal pathogens of Phytophthora root rot of citrus, and these species can also infect citrus fruit and tree trunks, causing brown rot and gummosis, respectively. *P. nicotianae* is most active during the warmer months, whereas *P. citrophthora* can be recovered year-round (Hao et al. 2016).

Management of Phytophthora root rot is especially critical in the first few years of a new citrus planting during orchard establishment. Additionally in California, all *Phytophthora* diseases of citrus, including root rot, brown rot, and gummosis, are subject to intense management due to recent export restrictions to China that were imposed after the detection of orange fruit with brown rot caused by *P. syringae* (Kleb.) Kleb. and *P. hibernalis* Carne that are quarantine pathogens in this country (Förster et al. 2016). Phytophthora root rot management requires an integration of rootstock selection, cultural practices, and fungicide applications. *Phytophthora*-tolerant rootstocks such as Trifoliata and Trifoliata hybrids (e.g., C-35 and

Swingle) can be used for grafting commercially acceptable citrus scions (Ferguson et al. 1990; Roose 2014). Irrigation and orchard management strategies that reduce over-watering will render the soil environment less favorable for infection by the motile zoospores, the main infective propagules of *Phytophthora* spp.

Two fungicide modes of action (i.e., FRAC codes; FRAC 2018) were used extensively for many years as the sole chemical treatments for the management of Phytophthora root rot of citrus, the phenylamides (e.g., metalaxyl, mefenoxam; FRAC code 4) and phosphonates (e.g., fosetyl-Al, potassium phosphite; FRAC code 33). Resistance to the phenylamides has been reported in populations of several species of *Phytophthora* since the 1980s, including *P. citricola*, *P. cryptogea*, *P. infestans*, *P. megasperma*, and *P. nicotianae* (Davidse et al. 1981; Ferrin and Kabashima 1991; Gisi and Cohen 1996; Hwang and Benson 2005; Stack and Millar 1985). On citrus, resistant *P. nicotianae* isolates were identified in over half of the citrus nurseries sampled in Florida (Timmer et al. 1998). Phosphonate resistance has developed less frequently. Isolates of *P. capsici*, *P. cinnamomi*, and *P. infestans* with reduced sensitivity have been reported (Cohen and Samoucha 1984; Veena et al. 2010; Wilkinson et al. 2001). We recently determined that 10–20% of isolates of *P. syringae* and *P. citrophthora* from California citrus orchards were less sensitive to potassium phosphite in vitro. Brown rot caused by these isolates could not be controlled using registered rates of potassium phosphite in field applications, and postharvest treatments with this chemical were significantly less effective than when fruit were inoculated with a sensitive isolate (Adaskaveg et al. 2017; Förster et al. 2016).

To manage Phytophthora diseases of citrus and prevent further resistance development to phenylamide and phosphonate fungicides, new effective Oomycota fungicides need to be identified. Four new compounds have recently become available for evaluation on citrus, each with a different mode of action that is also different from fungicides currently available for citrus. They have been registered in the United States for some time for the management of various foliar and soil-borne diseases caused by Oomycota pathogens on selected crops where they proved to be highly efficacious (Bittner and Mila 2016; Cortright et al. 2016; Jackson et al. 2010; Ji et al. 2014; Patel

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et al. 2015), and their biochemical modes of action have been identified. The thiazole carboxamide (FRAC code 22) ethaboxam inhibits mitosis and cell division (Uchida et al. 2005), the benzamide (FRAC code 43) fluopicolide destabilizes plasma membrane formation by delocalizing a spectrin-like protein (Toquin et al. 2006), the carboxylic acid amide (FRAC code 40) mandipropamid targets the cellulose synthase-like gene and inhibits pathogen cell wall synthesis (Blum et al. 2010), and oxathiapiprolin, the first fungicide in the isoxazoline class (FRAC code 49), inhibits an oxysterol binding protein and thereby inhibits many cellular processes (Pasteris et al. 2016). We previously determined that the four fungicides were highly inhibitory to mycelial growth of *P. nicotianae*, *P. citrophthora*, *P. syringae*, and *P. hibernalis* isolates from citrus in California (Gray et al. 2018). Furthermore, oxathiapiprolin and mandipropamid also inhibited sporangium and oospore formation as well as zoospore cyst germination using low concentrations, and oxathiapiprolin was also highly active against chlamydospore formation (Gray et al. 2018).

The objective of this study was to evaluate the efficacy of ethaboxam, fluopicolide, mandipropamid, and oxathiapiprolin against citrus root rot under field and greenhouse conditions using citrus trees or seedlings inoculated with *P. nicotianae* or *P. citrophthora*. For this, we determined the effects on soil population sizes of the pathogens, incidence of root infections, and we measured tree growth parameters including tree canopy size, trunk diameter, and fruit yield.

Materials and Methods

Field studies with navel orange trees inoculated with *P. nicotianae*. A field trial was established in September 2013 with 3-year-old 'Fukumoto' navel orange (*Citrus sinensis* (L.) Osbeck) trees grafted on 'Carrizo citrange' rootstock. The field with a fine sandy loam was located at the field station of the University of California, Riverside (UCR Agricultural Operations). Twelve trees were planted in each of 28 rows with spacing between rows of 6.4 m and between trees in a row of 2.9 m. Trees were planted within basins and were irrigated with a bubbler emitter for every tree. Trees were protected by trunk guards to prevent sunburn and rabbit damage. Pest and weed control, as well as fertilization, were performed according to standard agricultural practices for citrus.

Trees were inoculated with chlamydospores of *P. nicotianae* (isolate 2390, Table 1) at planting. For this, four 9-mm-diameter plugs from 10-day-old cultures on 10% clarified V8 (V8C) agar (Ribeiro et al. 1978) were transferred to 20 ml of 10% V8C broth in 10-cm petri dishes and incubated at 20°C in the dark for 6 weeks. Mycelial mats with chlamydospores were blended using a PowerGen 700 homogenizer (Fisher Scientific Inc., Pittsburgh, PA) equipped with a 32-mm-diameter generator probe at speed setting 1 for 1 min. The suspension was adjusted to 3.4×10^5 chlamydospores/ml with sterile deionized water (SDW), and 50 ml was evenly dispensed over the root ball of each tree at planting. Based on the average volume of a root ball with adhering soil of 3 liters, approximately 15 chlamydospores per gram of soil were used as inoculum, which is the threshold of causing severe Phytophthora root rot of citrus (Lutz and Menge 1986).

A randomized complete block design with seven four-tree replications was used for each treatment. The first fungicide application was conducted 5 weeks after inoculation in October 2013. Additional applications were done during feeder root flushes in June and

September 2014, and May 2015. Ethaboxam (Intego; Valent USA, Walnut Creek, CA), fluopicolide (Presidio; Valent USA), mandipropamid (Revus; Syngenta Crop Protection, Greensboro, NC), oxathiapiprolin (Orondis; Syngenta Crop Protection), mefenoxam (Ridomil Gold SL; Syngenta Crop Protection), and selected mixtures were applied at rates indicated in Table 2 with a total of 11 treatments. Fungicide solutions (500 ml) were applied as soil drenches to the basin of each tree. Trees were then irrigated for 1 h to move the chemicals into the root zone. Trees treated with water were used as controls.

Field studies with navel orange trees inoculated with *P. citrophthora*. A field trial was initiated in June 2016 at UCR Agricultural Operations to evaluate effective application rates of the new fungicides. Ten 1-year-old 'Fisher' navel orange trees, grafted on Carrizo citrange rootstock, were planted in each of 12 rows. Between-row spacing was 6.4 m and within-row spacing was 3.5 m. Growing conditions and agricultural practices were the same as in the first field trial.

Trees were inoculated in July 2016 (3 weeks after planting) and October 2016. *P. citrophthora*-colonized vermiculite-oat seed inoculum was prepared as described by Matheron and Mircetich (1985) with minor modifications. In brief, a volume ratio of 2:4:3 of oat seeds, vermiculite, and 10% regular V8 broth (Miller 1955) was mixed, 600 ml of the mixture was filled into 1-liter plastic containers, and containers were autoclaved for 45 min each on four consecutive days. Each container was inoculated with 10 10% V8C agar plugs of *P. citrophthora* cultures (isolates 5475 or 5476, Table 1), mixed by shaking, and incubated at 25°C for 4–6 weeks with weekly shaking and mixing of the contents. Inoculum of the two isolates was mixed in a 1:1 ratio, and 25 ml was buried 10 cm deep at each of two locations at the base of each tree around the root ball.

A randomized complete block design with six single-tree replications was used for each treatment. Fungicides were applied in July 2016 (2 weeks after inoculation) and in May 2017. Fungicides and rates used are listed in Table 2, and fungicides were applied as described above. Plants treated with water were used as controls.

Disease assessments. Phytophthora root rot incidence and *Phytophthora* propagule populations in rhizosphere soil were evaluated to assess the efficacy of treatments. In the *P. nicotianae* trial, feeder roots and rhizosphere soil (0.3–0.5 kg per tree) were sampled in early June and early September 2014 before the second and third fungicide applications, respectively, and in late June 2015 after the last application. Samples were taken from the middle two trees of each four-tree replication and were combined. In the *P. citrophthora* trial, roots and soil were sampled in December 2016 and July 2017 from each tree. Samples were collected at the dripline of each tree at a depth of ≤ 20 cm, placed in plastic bags, and processed the same day.

Feeder roots were carefully separated from soil, rinsed three times with deionized water, and dried on paper towels. Roots were cut into 1-cm-long sections using a sterilized razor blade, and 20 pieces were plated onto each of two plates of selective *Phytophthora* isolation medium (PARHFB-V8C; Ferguson and Jeffers 1999; Hao et al. 2018). When present, root pieces with visible lesions were selected. Plates were incubated at 25°C in the dark for 2–3 days. *P. nicotianae* colonies were identified by their typical growth pattern (Erwin and Ribeiro 1996), and representative colonies were subcultured and verified for species identity using species-specific TaqMan qPCR (Hao et al. 2018). *Phytophthora*

Table 1. Information on *Phytophthora nicotianae* and *P. citrophthora* isolates used to inoculate citrus plants in field and greenhouse studies

Species	Isolate ID	Origin	Year	Source	EC ₅₀ values for mycelial growth inhibition (µg/ml) ^y					
					Etha-boxam	Fluopicolide	Mandipropamid	Oxathiapiprolin	Mefenoxam	Potassium phosphite
<i>P. nicotianae</i>	2390 ^z	Tulare, CA	2001	Rhizosphere	0.003	0.095	0.005	0.0006	0.232	49.12
<i>P. citrophthora</i>	2440 ^z	Ventura, CA	NA	Rhizosphere	0.049	0.048	0.004	0.0015	0.091	7.58
<i>P. citrophthora</i>	5475	Riverside, CA	2015	Rhizosphere	0.090	0.037	0.005	0.0004	0.053	6.17
<i>P. citrophthora</i>	5476	Riverside, CA	2015	Rhizosphere	0.093	0.037	0.004	0.0005	0.061	6.23

^y Effective concentrations (EC₅₀ values) of fungicides to inhibit mycelial growth by 50% for ethaboxam, fluopicolide, mandipropamid, oxathiapiprolin, and mefenoxam was determined using the spiral gradient dilution method; whereas the agar dilution method was used for potassium phosphite.

^z EC₅₀ values for ethaboxam, fluopicolide, mandipropamid, oxathiapiprolin, and mefenoxam were reported in Gray et al. (2018).

root rot incidence was calculated as the percentage of infected root pieces of 20 pieces plated per plate.

Rhizosphere soil was mixed well, and for every treatment replicate, a 10-g aliquot was mixed with 90 ml SDW in a 250-ml flask containing three stainless steel beads (6 mm diameter) on a rotary shaker (G24 Environmental Incubator Shaker, New Brunswick Scientific, Edison, NJ) at 150 rpm for 40 min. Aliquots of 1 ml soil suspension were plated on triplicate plates of PARHFB-V8C medium using a sterilized glass spreader. Plates were rinsed with deionized water after 24 h at 25°C in the dark to remove excess soil, and then further incubated for 1–2 days. The number of *P. nicotianae* colonies on each plate was assessed, and propagule populations in the rhizosphere were calculated as CFU per gram of soil.

Tree growth measurements. Tree trunk diameter, canopy size, and fruit production were determined to further evaluate the effectiveness of fungicide treatments in the first field trial. Trunk diameter of every tree at 10 cm above the graft union was measured in February, July, and December 2015 using a caliper. Tree canopy size was measured in April 2015 and 2016 for one representative tree with a visually average canopy size for the four trees per replication (preferentially one of the two middle trees). For this, a digital image of each entire canopy was obtained with a blue tarp (2.44 × 2.44 m) as background. Pictures were taken at approximately the same distance (5.4 m) from each tree, and the tarp was also kept at the same distance. This was done under overcast conditions to minimize shadow effects on tree canopy estimation. Images were analyzed using Assess 1.0: Image Analysis Software for Plant Disease Quantification (American Phytopathological Society, St. Paul, MN) to calculate the two-dimensional tree canopy. The percentage of tree canopy area in the blue tarp area was computed by

defining the two areas by their distinct colors (green versus blue) in the software, and the canopy area of each tree (in m²) was calculated based on the tarp area of 5.96 m². Mature, commercial-grade orange fruit were harvested in December 2016 and 2017, and the number and weight of fruit per tree were determined.

Greenhouse studies with inoculated citrus plants. ‘Madam Vinous’ sweet orange that is considered susceptible to *Phytophthora* root rot was used in these studies. Plants were grown from seed in 1-liter pots in UC-C soil mix (Matkin and Chandler 1957) at 18–35°C in the greenhouse, watered using micro drippers, and fertilized with Osmocote 14-14-14 Slow Release Fertilizer (Scotts, Marysville, OH) once at transplanting. Six- to nine-month-old plants were inoculated with a chlamydospore suspension of *P. nicotianae* (isolate 2390, Table 1) that was produced as described above. One milliliter suspension (3,000 chlamydospores/ml) was added into each of four holes (3 cm deep, 7 mm diameter) around each plant resulting in an equivalent of approximately 20 chlamydospores/g soil.

Treatments were applied 1 week after inoculation. For potassium phosphite, ProPhyt (Helena Chemical Co., Collierville, TN) was used. Greenhouse application rates (Table 2) were proportionally reduced from the respective field rates based on the ratio of the average surface of tree basins in the field to the soil surface of a potted plant (i.e., a 25:1 ratio). A randomized complete block design with four single-pot replications was used for each treatment. Fungicides were applied as aqueous suspensions (50 ml/plant) to the soil around each plant. Plants treated with 50 ml of water were used as controls.

The effectiveness of fungicides was evaluated after 6–7 months. For each plant, the root ball with soil was carefully removed from the pot. Soil adjacent to roots was collected, and root balls were

Table 2. Fungicide treatments evaluated to manage *Phytophthora* root rot of navel orange under field and greenhouse conditions

Treatment	Field studies				Greenhouse studies		
	Rate		<i>P. nicotianae</i> study	<i>P. citrophthora</i> study	Rate (mg / pot) ^a	<i>P. nicotianae</i> study	<i>P. citrophthora</i> study
	g / ha	g / tree ^u					
Mefenoxam	246	0.71	X ^{v,w}	— ^v	—	—	—
	561	1.62	X ^w	—	67	—	X
	1,122	3.24	—	—	134	X	—
	2,245	6.48	X ^w	—	—	—	—
Ethaboxam	18	0.05	—	X	—	—	—
	35	0.10	—	X	—	—	—
	70	0.20	—	X	—	—	—
	140	0.41	—	X	—	—	—
	351	1.01	X	—	41	X	X
	702	2.03	—	—	81	X	—
	1,404	4.06	—	—	—	—	—
Fluopicolide	35	0.10	—	X	—	—	—
	70	0.20	—	X	—	—	—
	140	0.41	X	X	14	X	X
	210	0.61	X	—	24	X	—
	281	0.81	—	X	—	—	—
Mandipropamid	18	0.05	—	X	—	—	—
	37	0.11	—	X	—	—	—
	73	0.21	—	X	—	—	—
	146	0.42	X	X	17	X	X
	292	0.84	—	—	34	X	—
Oxathiapirolin	18	0.05	—	X	—	—	—
	35	0.10	—	X	—	—	—
	70	0.20	X	X	8	X	X
	140	0.41	X	X	16	X	—
Potassium phosphite	—	—	—	—	201 ^x	X	X
Ethaboxam + fluopicolide	351 + 210	1.01 + 0.61	X	—	—	—	—
Fluopicolide + mefenoxam	210 + 1,122	0.61 + 3.24	X ^y	—	—	—	—
Mefenoxam + oxathiapirolin	1,122 + 70	3.24 + 0.20	X ^z	—	—	—	—
Mefenoxam + oxathiapirolin	1,122 + 140	3.24 + 0.41	X ^y	—	—	—	—

^u Field rates per tree were calculated based on 346 trees/ha, greenhouse rates were calculated from field rates based on the soil area per tree:soil area per pot = 25:1.

^v — = Fungicide at the selected rate was not evaluated in the study, X = Fungicide at the selected rate was evaluated in the study.

^w Mefenoxam was used at 246 g/ha in the first application, at 561 g/ha in the second and third applications, and at 2,245 g/ha in the last application.

^x Recommended label rate for citrus seedlings was used (31.3 ml potassium phosphite/liter soil).

^y Mefenoxam was applied at 246 g/ha in the first three applications.

^z Mefenoxam was applied at 123 g/ha in the first three applications.

Table 3. Univariate repeated-measures analysis of variance (ANOVA)^w of *Phytophthora* root rot incidence, *Phytophthora* propagules per gram soil, and tree trunk diameter of navel orange infected by *Phytophthora nicotianae* in field studies

Parameter	Source of variation	df	Type III sum of squares	F value	P value	Mauchly's test of sphericity P value	Adjusted P value by G-G ^x
Root rot incidence ^y	Block	6	57.5	0.6333	0.7031		
	Error (block)	66	998.9				
	Treatment	11	4391.5	26.3774	<0.0001		
	Error (treatment)	66	998.9				
	Time	2	376.8	8.4366	0.0004	0.0003	0.0009
	Error (time)	132	2948.1				
	Block × time	12	201.2	0.7506	0.6996	0.0003	0.6735
	Error (block × time)	132	2948.1				
	Treatment × time	22	1402.7	2.8548	0.0001	0.0003	0.0004
CFU per gram soil ^{y,z}	Error (treatment × time)	132	2948.1				
	Block	6	11691	0.7639	0.6009		
	Error (block)	66	168340				
	Treatment	11	563615	20.0885	<0.0001		
	Error (treatment)	66	168340				
	Time	2	24906	7.2457	0.0010	0.0144	0.0017
	Error (time)	132	226861				
	Block × time	12	15415	0.7475	0.7028	0.0144	0.6873
	Error (block × time)	132	226861				
Tree trunk diameter ^y	Treatment × time	22	103441	2.7358	0.0002	0.0144	0.0004
	Error (treatment × time)	132	226861				
	Block	6	4637	11.4623	<0.0001		
	Error (block)	318	21439				
	Treatment	11	2553	3.4424	0.0002		
	Error (treatment)	318	21439				
	Time	2	31654	1675.1265	<0.0001	<0.0001	<0.0001
	Error (time)	636	6009				
	Block × time	12	881	7.7738	<0.0001	<0.0001	<0.0001
Tree canopy ^y	Error (block × time)	636	6009				
	Treatment × time	22	692	3.3289	<0.0001	<0.0001	<0.0001
	Error (treatment × time)	636	6009				
	Block	6	5.96	6.6357	<0.0001		
	Error (block)	66	9.87				
	Treatment	11	4.19	2.5445	0.0010		
	Error (treatment)	66	9.87				
	Time	1	122.45	1276.2644	<0.0001		
	Error (time)	66	6.33				
Fruit number per tree ^y	Block × time	6	1.72	2.9921	0.0120		
	Error (block × time)	66	6.33				
	Treatment × time	11	1.25	1.1816	0.3169		
	Error (treatment × time)	66	6.33				
	Block	4	20262	4.3327	0.0049		
	Error (block)	44	51443				
	Treatment	11	63765	4.9581	<0.0001		
	Error (treatment)	44	51443				
	Time	1	514568	376.2906	<0.0001		
Fruit weight per tree ^y	Error (time)	44	60169				
	Block × time	4	4933	0.9018	0.4712		
	Error (block × time)	44	60169				
	Treatment × time	11	33472	2.2252	0.0301		
	Error (treatment × time)	44	60169				
	Block	4	1448	2.3448	0.0693		
	Error (block)	44	6795				
	Treatment	11	8250	4.8567	<0.0001		
	Error (treatment)	44	6795				
	Time	1	6706	922.4177	<0.0001		
	Error (time)	44	6692				
	Block × time	4	718	1.1800	0.3328		
	Error (block × time)	44	6692				
	Treatment × time	11	5928	3.5432	0.0013		
	Error (treatment × time)	44	6692				

^w Univariate repeated-measures ANOVA was performed in R (3.1.3).

^x P value was adjusted using Greenhouse-Geisser correction.

^y For parameters that were measured three times during the trial period, Mauchly's test was performed for sphericity. When sphericity was violated ($P < 0.05$), adjusted P values were used to determine the significance of variance. For tree canopy, fruit number, and fruit weight, only two repeated measures were done.

^z Colony forming units (CFU) were determined by soil plating.

rinsed with water. Soil and feeder roots were plated on duplicate plates of PARHFB-V8C medium as described above for field studies to assess root rot incidence and *P. nicotianae* propagule soil populations. This experiment was done twice.

In another experiment, 10- to 12-month-old plants were inoculated with *P. citrophthora* (isolate 2440, Table 1) that was grown on long-grain white rice (Holmes and Benson 1994) for 4 weeks. For inoculation, two colonized rice grains were buried 3 cm deep at each of four locations around each plant (8 grains/pot). Fungicides (Table 2) were applied as described above after 1 week. A randomized complete block design with four single-pot replications was used for each treatment. Plants treated with water were used as controls. After 4–5 months, the efficacy of fungicides was evaluated as described above. This experiment was repeated.

Statistical analysis. For field trials, data for *Phytophthora* root rot incidence and *Phytophthora* propagule populations in soil, tree trunk diameters, tree canopy sizes, and fruit production at different evaluation times were subjected to a repeated measures univariate analysis

of variance (ANOVA) to determine the effect of fungicide treatments over the trial periods. Mauchly's test for sphericity was performed to test the equality of variance of the differences between all combinations of factors at each measurement time when more than two repeated measurements were conducted. When sphericity was violated ($P < 0.05$), valid P values were obtained using the Greenhouse-Geisser correction. Differences between means of treatments at each measurement time were analyzed using Fisher's least significant difference (LSD) test, and differences between the means of the control and fungicide treatments were analyzed using pairwise t tests.

For repeated greenhouse experiments, the homogeneity of variances for *Phytophthora* root rot incidence and *Phytophthora* propagule populations in soil were tested using Bartlett's test of homogeneity. Homogeneous data were combined and analyzed using ANOVA, and the differences between means of treatments were compared using a pairwise t test and Fisher's LSD test.

All statistical analyses were performed in R (version 3.1.3; R Core Team 2015) using the agricolae (De Mendiburu 2015) and car (Fox

Fig. 1. Efficacy of fungicides in reducing **A**, incidence of *Phytophthora* root rot of navel orange on 'Carrizo citrange' rootstock caused by *Phytophthora nicotianae* and **B**, pathogen populations in the soil in a field trial initiated in September 2013. Fungicides were applied in October 2013, June and September 2014, and May 2015. Mefenoxam used by itself was applied at 246 g/ha in the first application, at 561 g/ha in the second and third applications, and at 2,245 g/ha in the fourth application. In the fluopicolide-mefenoxam and mefenoxam-oxathiapirolin (140 g/ha) mixtures, mefenoxam was applied at 246 g/ha in the first three applications, whereas in the mefenoxam-oxathiapirolin (70 g/ha) mixture, mefenoxam was applied at 123 g/ha in the first three applications. Feeder roots and rhizosphere soil were sampled in June and September 2014 (before the second and third fungicide applications, respectively), and June 2015 (after the fourth application). Bars followed by the same letter do not differ significantly according to Fisher's least significance difference test at $P = 0.05$.

and Weisberg 2011) packages. Results were considered significant at $P \leq 0.05$.

Results

Efficacy of new fungicides against *Phytophthora* root rot of navel orange caused by *P. nicotianae* in field studies. There were significant main effects of sampling time for *Phytophthora* root rot incidence ($P = 0.0009$) and *P. nicotianae* propagule soil populations ($P = 0.0017$) and a significant interaction between sampling time and fungicide treatment on the incidence of *Phytophthora* root rot ($P = 0.0004$) and *P. nicotianae* propagules in soil ($P = 0.0004$) (Table 3). Seven months after the first fungicide application (June 2014), all fungicide treatments (including mixtures), except mefenoxam by itself, significantly ($P \leq 0.0002$) reduced the incidence of *Phytophthora* root rot to low levels as compared with the control (Fig. 1A). Three months after the second application (September 2014), treatments with the new fungicides reduced the incidence to zero or near zero levels. Mefenoxam was only effective after the rate was increased to 2,245 g/ha at the fourth application (June 2015). Fungicide effects on soil population sizes of *P. nicotianae* were very similar as for root rot incidence, and populations were dramatically decreased by treatments containing the new compounds starting after the first application (Fig. 1B).

For tree trunk diameters, a significant effect of measurement time ($P < 0.0001$) and a significant interaction between measurement time and treatment ($P < 0.0001$) were found (Table 3). Differences in trunk diameters among treated and control trees were observed in July and December 2015 (Table 4) after the fourth fungicide application. In July, diameters were significantly larger ($P \leq 0.0424$) after treatments containing fluopicolide or oxathiapiprolin, and in December, ethaboxam-treated trees also showed increased trunk diameters. In July and December evaluations, trunk diameters after mandipropamid or mefenoxam treatments by themselves, however, were similar ($P \geq 0.1496$) to those of control trees.

Two-dimensional tree canopy sizes were significantly different among treatments ($P < 0.0010$) and between measurement times ($P < 0.0001$) (Table 3). In April 2015 and 2016, canopy sizes were significantly larger in comparison with control trees after treatment with the lower and higher rates of fluopicolide ($P = 0.0024$ and $P = 0.0199$, or $P = 0.0391$ and $P = 0.0498$, for April 2015 and 2016, respectively), the lower and higher rates of oxathiapiprolin ($P = 0.0427$

and $P = 0.0426$, or $P = 0.0334$ and $P = 0.0428$, respectively), or the ethaboxam-fluopicolide mixture ($P = 0.0242$ and $P = 0.0020$, respectively) (Table 4). For other treatments, no differences in canopy size as compared with the control were found in both evaluations (ethaboxam: $P = 0.2766$ and $P = 0.0993$; mandipropamid: $P = 0.3113$ and $P = 0.2716$; mefenoxam: $P = 0.9289$ and $P = 0.6927$; mefenoxam mixed with a low rate of oxathiapiprolin: $P = 0.1071$

Table 5. Univariate repeated-measures analysis of variance (ANOVA)^x for *Phytophthora* root rot incidence and *Phytophthora* propagules per gram of soil in a field study with *Phytophthora citrophthora*-infected navel orange trees

Parameter	Source of variation	df	Type III sum of squares	F value	P value
Root rot incidence ^y	Block	5	394.6	2.6211	0.0302
	Error (block)	80	2409.0		
	Treatment	16	2000.9	4.1530	<0.0001
	Error (treatment)	80	2409.0		
	Time	1	717.2	26.2220	<0.0001
	Error (time)	80	2188.1		
	Block × time	5	326.0	2.3839	0.0455
	Error (block × time)	80	2188.1		
	Treatment × time	16	834.4	1.9067	0.0316
	Error (treatment × time)	80	2188.1		
CFU per gram soil ^{y,z}	Block	5	291331	1.0837	0.3758
	Error (block)	80	4301141		
	Treatment	16	4410695	5.1274	<0.0001
	Error (treatment)	80	4301141		
	Time	1	3047815	56.9863	<0.0001
	Error (time)	80	4278662		
	Block × time	5	292411	1.0935	0.3706
	Error (block × time)	80	4278662		
	Treatment × time	16	3463375	4.0473	<0.0001
	Error (treatment × time)	80	4278662		

^x Univariate repeated-measures ANOVA was performed in R (3.1.3).

^y All parameters were measured twice during the field trial period.

^z Colony forming units (CFU) were determined by soil plating.

Table 4. Tree trunk diameter, tree canopy size, and fruit production of navel orange trees treated with fungicides to manage *Phytophthora* root rot caused by *Phytophthora nicotianae*

Treatment (g / ha)	Tree trunk diameter (mm) ^u			Tree canopy (m ²) ^v		Fruit production			
						Dec 2016		Dec 2017	
	Feb 2015	Jul 2015	Dec 2015	Apr 2015	Apr 2016	Fruit no. / tree	Fruit weight (kg) / tree	Fruit no. / tree	Fruit weight (kg) / tree
Control	26.6 ab ^w	31.2 cd	36.1 c	0.5 c	1.9 d	56 d	13.0 c	144 cd	60.5 de
Mefenoxam (2,245) ^x	24.0 c	30.6 d	35.8 c	0.5 c	2.0 cd	60 cd	13.1 c	122 d	53.6 e
Ethaboxam (351)	27.3 ab	33.5 bc	39.6 b	0.6 bc	2.4 abcd	68 bcd	15.3 bc	219 ab	89.2 abc
Fluopicolide (140)	27.4 ab	35.5 ab	41.8 ab	0.7 ab	2.5 abc	81 abc	16.8 abc	242 ab	100.4 ab
Fluopicolide (210)	26.7 ab	35.1 ab	41.8 ab	0.8 a	2.6 ab	92 a	19.9 a	217 ab	88.1 abc
Mandipropamid (146)	25.2 bc	33.0 bcd	38.7 bc	0.6 bc	2.2 bcd	72 abcd	16.6 abc	239 ab	103.2 a
Oxathiapiprolin (70)	25.5 abc	35.4 ab	41.6 ab	0.7 ab	2.5 abc	88 ab	18.8 ab	259 a	99.8 ab
Oxathiapiprolin (140)	27.0 ab	35.2 ab	40.9 b	0.7 ab	2.4 abc	72 abcd	15.7 bc	242 ab	100.8 ab
Ethaboxam + fluopicolide (351 + 210)	27.3 ab	36.7 a	44.3 a	0.7 ab	2.8 a	90 a	18.9 ab	196 bc	85.8 abc
Fluopicolide + mefenoxam (210 + 1,122) ^y	26.9 ab	35.3 ab	40.8 b	0.7 ab	2.4 abcd	80 abc	17.6 ab	196 bc	79.8 bcd
Mefenoxam + oxathiapiprolin (1,122 + 70) ^z	27.7 a	34.1 ab	40.4 b	0.6 b	2.3 bcd	73 abcd	15.5 bc	196 bc	75.1 cde
Mefenoxam + oxathiapiprolin (1,122 + 140) ^y	27.3 ab	34.5 ab	41.4 ab	0.7 ab	2.4 abcd	76 abcd	15.9 abc	208 ab	81.3 abcd

^u Tree trunk diameter was measured 10 cm above the graft union.

^v The two-dimensional tree canopy area was calculated as percent area covered by the tree canopy of a blue standard background using Assess 1.0.

^w Numbers followed by the same letter do not differ significantly according to Fisher's least significance difference test at $P = 0.05$.

^x Mefenoxam was used at 246 g/ha in the first application, at 561 g/ha in the second and third applications, and at 2,245 g/ha in the last application.

^y Mefenoxam was applied at 246 g/ha in the first three applications.

^z Mefenoxam was applied at 123 g/ha in the first three applications.

and $P = 0.2007$, respectively). A significant increase in canopy size was only observed in April 2015 for fluopicolide mixed with mefenoxam ($P = 0.0172$ and $P = 0.0770$, for 2015 and 2016, respectively); and for mefenoxam mixed with a higher rate of oxathiapiprolin ($P = 0.0332$ and $P = 0.0944$, respectively). No phytotoxic effects to trees such as leaf burn were observed for any of the treatments.

Fruit production was assessed in December 2016 and 2017. A significant effect of evaluation time ($P < 0.0001$ for fruit number per tree and $P < 0.0001$ for fruit weight per tree) and a significant interaction between evaluation time and treatment ($P = 0.0301$ for fruit number and $P = 0.0013$ for fruit weight) were observed (Table 3). In both years, significant increases in fruit number and weight as compared with control trees were observed after treatment with the 210-g/ha rate of fluopicolide ($P = 0.0058/0.0047$ and $P = 0.0239/0.0173$ for fruit number/fruit weight in December 2016 and 2017 harvests, respectively), the 70-g/ha rate of oxathiapiprolin ($P = 0.0120/0.0164$ and $P = 0.0006/0.0010$, respectively), and the ethaboxam-fluopicolide mixture ($P = 0.0082/0.0140$ and $P = 0.0222/0.0274$, respectively) (Table 4). In the 2017 evaluation, 4 years after orchard establishment, the number of fruit per tree was significantly higher ($P \leq 0.0239$) for all single-active-ingredient treatments except for mefenoxam used by itself ($P = 0.4876$). At this time, fruit weight per tree was significantly ($P \leq 0.0173$) increased by all single-active-ingredient treatments except for mefenoxam ($P = 0.5412$), the fluopicolide-mefenoxam mixture ($P = 0.0904$), and the mefenoxam (1,122 g/ha)-oxathiapiprolin (140 g/ha) mixture ($P = 0.0689$). The highest yields were obtained after treatment with mandipropamid (103.2 kg/tree; as compared with 60.5 kg for control trees), the 140-g/ha rate of fluopicolide (100.4 kg/tree), and oxathiapiprolin (99.8 and 100.8 kg/tree for the 70-g and 140-g/ha rates, respectively).

Evaluation of fungicide application rates to manage *Phytophthora* root rot of navel orange caused by *P. citrophthora* in field studies. There were significant main effects of sampling time

for root rot incidence ($P < 0.0001$) and *P. citrophthora* propagule soil populations ($P < 0.0001$) and a significant interaction between sampling time and fungicide treatment on the incidence of root rot ($P = 0.0316$) and *P. citrophthora* propagules in soil ($P < 0.0001$) (Table 5). In an evaluation of root rot incidence after the first treatment (December 2016), applications with ethaboxam and mandipropamid resulted in rate-dependent differences in efficacy, and higher rates were generally more effective (Fig. 2). For ethaboxam, only the highest rate (140 g/ha, $P = 0.0299$), and for mandipropamid, the two highest rates (73 g/ha and 146 g/ha, $P = 0.0112$ and $P = 0.0086$, respectively) evaluated resulted in a significant difference in root rot incidence from the control. For fluopicolide and oxathiapiprolin, there was no significant difference among rates ($P \geq 0.7019$ for 35 g to 281 g fluopicolide/ha; and $P \geq 0.2939$ for 18 g to 140 g oxathiapiprolin/ha), and all four rates evaluated were highly effective ($P \leq 0.0186$ for fluopicolide, $P \leq 0.0086$ for oxathiapiprolin). In the second evaluation in July 2017, all treatments, except an intermediate rate of ethaboxam were statistically similarly effective in reducing the incidence of root rot (Fig. 2). Results for *P. citrophthora* soil populations mostly reflected those for root rot incidence. However, fluopicolide also showed rate-dependent differences, with the highest rate of 281 g/ha being the most effective ($P = 0.0145$) in the first evaluation in December 2016 (Fig. 2).

Evaluation of new fungicides against *Phytophthora* root rot of orange in greenhouse studies. Variances of data from repeated experiments were homogeneous and therefore, results presented are the mean of two experiments. Similar to the field studies, ethaboxam, fluopicolide, mandipropamid, and oxathiapiprolin effectively reduced the incidence of root rot ($P < 0.0001$) and soil population sizes of *P. nicotianae* ($P < 0.0001$) to zero or near zero levels (Fig. 3). There was no significant difference in efficacy between the two rates of each fungicide evaluated ($P \geq 0.79$ for root rot incidence, and $P \geq 0.8805$ for *P. nicotianae* soil populations, respectively). Mefenoxam was significantly less effective in reducing soil populations than the

Fig. 2. Efficacy of selected rates of new Oomycota fungicides in reducing the incidence of *Phytophthora* root rot of navel orange on 'Carrizo citrange' rootstock caused by *Phytophthora citrophthora* and pathogen populations in the soil in a field trial initiated in June 2016. Fungicides were applied in July 2016 and May 2017. Feeder roots and rhizosphere soil were sampled in December 2016 and July 2017. Bars followed by the same letter do not differ significantly according to Fisher's least significance difference test at $P = 0.05$.

other fungicides, but populations were still significantly ($P < 0.0001$) lower as compared with the control. Potassium phosphite was also highly effective using the label rate for nursery use and significantly ($P < 0.0001$) reduced root rot incidence and *P. nicotianae* propagules in the soil. No phytotoxicity was observed on plants with any of the treatments at the rates used, but higher rates of mefenoxam and potassium phosphite used in other studies caused stunting and dieback (data not presented).

In another greenhouse study, orange plants were inoculated with *P. citrophthora* after soil treatment. Applications with fungicides were done at the lower of the two rates used in the greenhouse study above with *P. nicotianae*. Although disease incidence in the untreated control was low, significant reductions ($P < 0.0001$ for root rot incidence and *P. citrophthora* populations) were obtained using ethaboxam, fluopicolide, mandipropamid, or oxathiapiprolin, and root rot incidence and soil populations were reduced to zero or very low levels (Fig. 4). Mefenoxam and potassium phosphite were effective against root rot and showed moderate activity in reducing pathogen soil populations.

Discussion

This is the first study evaluating the new Oomycota fungicides ethaboxam, fluopicolide, mandipropamid, and oxathiapiprolin for managing Phytophthora root rot of citrus. Among soil-borne diseases of citrus, Phytophthora root rot is the most serious, occurring in most growing regions worldwide, whereas others including Armillaria root rot (caused by *Armillaria* spp.), dry root rot (caused by *Fusarium solani*), and Rosellinia root rot (caused by *Rosellinia necatrix*) are of only localized importance (Adaskaveg et al. 2014). Therefore, effective management strategies for Phytophthora root rot need to be available, also in light of increasing restrictions on the use of soil fumigants for preparation of new planting sites. Moreover, there is some evidence for a positive interaction between infection levels by huanglongbing (HLB) and Phytophthora root rot and for an enhancement of HLB-induced symptoms in *P. nicotianae*-infected citrus plants (Graham et al. 2011, 2013). This further stresses the significance of our study.

In comparative studies with the current commercial standards, mefenoxam and potassium phosphite, we demonstrated the superior effectiveness of ethaboxam, fluopicolide, mandipropamid, and

oxathiapiprolin in reducing *Phytophthora* spp. soil populations and root rot in field and greenhouse studies as well as increasing crop yield. Our field studies established fungicide effectiveness under California orchard conditions using a common commercial irrigation system that exposes the tree rhizosphere to regular wetness conditions and potential infection periods for the root rot pathogens. In the field study with *P. citrophthora*, we identified lower highly effective rates for mandipropamid (73 g versus 146 g/ha) and oxathiapiprolin (35 g versus 70 g/ha) when results were compared with the first field study on root rot caused by *P. nicotianae* that used rates originally recommended by the respective registrants. Fungicide rate calculations for plants in the greenhouse were based on pot surface area in comparison with tree trunk basin area in the field. Although the greenhouse-applied rates may still not be equivalent to labeled field rates, these studies demonstrated postinfection activity of the fungicides. This is important because the disease in a newly planted field may be initiated from infected nursery stock.

The high efficacy of ethaboxam, fluopicolide, mandipropamid, and oxathiapiprolin in reducing disease and *Phytophthora* soil populations correlated with their low in vitro effective concentration (EC) values against mycelial growth of several *Phytophthora* species from citrus that we determined previously (Gray et al. 2018). The isolates of *P. nicotianae* and *P. citrophthora* in the current study were used as representatives of each species with EC₅₀ values for each fungicide within the baseline range of sensitivity. In the Gray et al. (2018) study, values for oxathiapiprolin were the lowest among the four compounds for inhibition of five *Phytophthora* life stages including mycelial growth, sporangium formation, zoospore cyst germination, and chlamydospore and oospore formation. This high toxicity was reflected by low effective field rates (i.e., 35 g/ha) that we identified in the field study with *P. citrophthora*.

Ethaboxam (Cortright et al. 2016; Kim et al. 2004), fluopicolide and mandipropamid (Cerkaskas et al. 2015; Foster and Hausbeck 2010; Jackson et al. 2010; Jiang et al. 2015; Meyer and Hausbeck 2013; Shin et al. 2010), and oxathiapiprolin (Bittner and Mila 2016; Ji and Csinos 2015; Ji et al. 2014; Miao et al. 2016) were previously shown to provide a high level of control for a range of foliar and root diseases of field and vegetable crops caused by species of *Phytophthora*. However, our study is the first validation of the effective use of the new compounds on a perennial tree crop, and the results presented are facilitating their registration on citrus. We conducted our studies using orange trees inoculated with *P. nicotianae* or *P. citrophthora*, the main *Phytophthora* root rot pathogens

Fig. 3. Efficacy of new Oomycota fungicides against *Phytophthora* root rot caused by *Phytophthora nicotianae* in greenhouse studies with 'Madam Vinous' sweet orange seedlings. Fungicides were applied 1 week after soil inoculation. The incidence of *Phytophthora* root rot and pathogen soil populations were evaluated after 7 months. Data are the averages of two experiments. Bars followed by the same letter do not differ significantly according to Fisher's least significance difference test at $P = 0.05$.

Fig. 4. Efficacy of new Oomycota fungicides against *Phytophthora* root rot caused by *Phytophthora citrophthora* in greenhouse studies with 'Madam Vinous' sweet orange seedlings. Fungicides were applied 1 week after soil inoculation. The incidence of *Phytophthora* root rot and pathogen soil populations were evaluated after 5 months. Data are the averages of two repeated experiments. Bars followed by the same letter do not differ significantly according to Fisher's least significance difference test at $P = 0.05$.

in California (Hao et al. 2016). Additional species are major causal agents in other citrus growing areas, for example *P. palmivora* in Florida (Graham and Menge 1999), and further studies using these species may be warranted.

In comparison with the four new fungicides, effectiveness of potassium phosphite in greenhouse studies was high to moderate and was moderate for mefenoxam. In the field study, a significant reduction in disease and soil populations by mefenoxam was only observed after increasing applications to high label rates. Lower rates were initially used because this fungicide is known to cause phytotoxic effects to young citrus trees as was also observed in preliminary greenhouse studies (data not presented here) where a range of rates was evaluated. Thus, the reduced effectiveness of mefenoxam in our study, a treatment that has been used successfully in commercial applications for managing *Phytophthora* root rot of various tree crops for many years, may have been due to using inadequate rates. Furthermore, trees were inoculated with an isolate of *P. nicotianae* with an EC₅₀ value for mycelial growth of 0.24 mg/liter that was in the midrange among 31 isolates from California evaluated previously (Gray et al. 2018). Because baseline sensitivities before commercial use of mefenoxam were never established for *P. nicotianae* from citrus, and the phenylamide class of fungicides has been used since the 1980s in California citriculture, this isolate may be part of a less-sensitive subpopulation of the species that cannot be easily managed with mefenoxam applications.

Soil populations of untreated control trees in our field and greenhouse studies were often very high considering that >15 propagules/g of soil is considered a threshold level where management is recommended (Adaskaveg et al. 2014; Lutz and Menge 1986). Still, disease incidence of feeder roots was mostly low, especially during summer samplings in the field. We chose Carrizo citrange in the field studies because it is commonly used commercially as a rootstock. It is considered of intermediate susceptibility (Ferguson et al. 1990) or tolerant (Graham and Menge 1999) to *Phytophthora* root rot, and this could have accounted for the low disease incidence. In the greenhouse studies, disease incidence may have been increased by pruning feeder roots of seedlings as it was done in studies by others (Graham 1995). Root injuries may occur naturally in the field by nematode or root weevil (*Diaprepes* spp.) infestations in the soil, and these pests are known to increase the incidence of *Phytophthora* root rot (Graham and Menge 1999; Graham et al. 2003). Still, although disease incidence was overall low in our studies, fungicide efficacy could be compared and significant differences were observed.

The four new Oomycota fungicides are single-site mode of action inhibitors (Blum et al. 2010; Pasteris et al. 2016; Toquin et al. 2006; Uchida et al. 2005). Their resistance risk currently has not been completely characterized (FRAC 2018), and resistant field isolates have not yet been detected in *Phytophthora* species (Gisi and Sierotzki 2015; Lu et al. 2011; Saville et al. 2015). Resistance, however, has been described for *Plasmopara viticola*, another Oomycota organism, to mandipropamid (Gisi et al. 2007). In our previous baseline sensitivity assessments, outliers with higher EC₅₀ values for mycelial growth inhibition of *P. syringae* by fluopicolide and of *P. citrophthora* by ethaboxam were identified that were ≥23-fold less sensitive than the most sensitive isolates of the respective species used in the study, and this was considered to possibly indicate a potential for selecting isolates with reduced sensitivity to these fungicides (Gray et al. 2018). Thus, as with any single-site mode of action fungicide, resistance management strategies should be followed from the onset of commercial use. Because two of the new fungicides each have the same registrant in the United States, the commercialization of pre-mixtures will be facilitated.

In summary, our study demonstrated that the new Oomycota fungicides ethaboxam, fluopicolide, mandipropamid, and oxathiapiprolin provided highly effective control of *Phytophthora* root rot of citrus caused by *P. nicotianae* or *P. citrophthora*. The efficacy was generally better than for the previously available fungicides mefenoxam and potassium phosphite. The new compounds promoted the recovery of infected trees and enhanced fruit yield, with fluopicolide and oxathiapiprolin showing the most consistent increases in

these measures. Based in part on our studies, fluopicolide recently received a federal and oxathiapiprolin a full registration for use on citrus, whereas registration for ethaboxam and mandipropamid has been requested.

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