Baseline Sensitivities of New Fungicides and Their Toxicity to Selected Life Stages of *Phytophthora* Species from Citrus in California

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Abstract

Phytophthora citrophthora, *P. syringae*, *P. nicotianae*, and *P. hibernalis* are important pathogens of citrus in California but few chemical treatments are currently available. In vitro toxicities of four new fungicides to isolates of *Phytophthora* spp. from California were determined. Mean effective concentration values to inhibit mycelial growth by 50% for ethaboxam, fluopicolide, mandipropamid, oxathiapiprolin, and mefenoxam were 0.068, 0.04, 0.004, 0.0003, and 0.039 $\mu\text{g/ml}$, respectively, for 62 isolates of *P. citrophthora*; 0.005, 0.045, 0.003, 0.0001, and 0.008 $\mu\text{g/ml}$, respectively, for 71 isolates of *P. syringae*; 0.016, 0.057, 0.005, 0.0005, and 0.183 $\mu\text{g/ml}$, respectively, for 31 isolates of *P. nicotianae*; and 0.030, 0.018, 0.005, <0.0003, and ≤ 0.001 $\mu\text{g/ml}$, respectively, for two isolates of *P. hibernalis*. Mean values for $\geq 90\%$ inhibition of sporangia formation

of four isolates of *P. citrophthora* were 0.1, 0.28, 0.026, 0.005, and 55 $\mu\text{g/ml}$ for ethaboxam, fluopicolide, mandipropamid, oxathiapiprolin, and mefenoxam, respectively. Zoospore cyst germination of *P. citrophthora* was most inhibited by oxathiapiprolin and mandipropamid. Chlamydospore formation of *P. nicotianae* was most sensitive to oxathiapiprolin, with a mean $\geq 90\%$ reduction (EC_{50}) of 0.002 $\mu\text{g/ml}$, moderately sensitive to mandipropamid ($\text{EC}_{50} = 0.2$ $\mu\text{g/ml}$) and mefenoxam ($\text{EC}_{50} = 0.6$ $\mu\text{g/ml}$), and least sensitive to ethaboxam and fluopicolide ($\text{EC}_{50} = 1$ $\mu\text{g/ml}$). Oospore formation of *P. nicotianae* was inhibited by $\geq 90\%$ using oxathiapiprolin at 0.0004 $\mu\text{g/ml}$, mandipropamid at 0.02 $\mu\text{g/ml}$, ethaboxam at 0.1 $\mu\text{g/ml}$, or fluopicolide at 0.4 $\mu\text{g/ml}$, whereas 62% inhibition was obtained by mefenoxam at 40 $\mu\text{g/ml}$.

Phytophthora spp. are plant pathogens in the kingdom Stramenopila that cause diseases on a wide variety of cultivated and noncultivated plants. Numerous species have been associated with citrus crops worldwide (Erwin and Ribeiro 1996), and in California, *Phytophthora citrophthora* (R. E. Sm. & E. H. Sm.) Leonian, *P. syringae* (Kleb.) Kleb., *P. nicotianae* Breda de Haan (syn. *P. parasitica* Dastur), and *P. hibernalis* Carne are present (Adaskaveg et al. 2014). These species can cause fruit brown rot (all four species), root rot (*P. nicotianae* and *P. citrophthora*), foot rot (*P. nicotianae*), and trunk cankers or gummosis (all four species) (Erwin and Ribeiro 1996; Graham and Menge 2000). Overall, *P. hibernalis* is the least commonly encountered species in California. *P. nicotianae* is most prevalent from late spring to early fall, *P. citrophthora* can be found year-round, whereas *P. syringae* and *P. hibernalis* are restricted to the cooler seasons (Hao et al. 2016a, 2018). Recently, *P. syringae* and *P. hibernalis* have been designated quarantine pathogens in China after they were detected in citrus fruit shipments from California. This has restricted the California citrus trade with this important export market, causing high economic losses to the state's citrus industry (Adaskaveg and Förster 2014).

The disease phases of *Phytophthora* spp. on citrus are closely interrelated. For example, a high incidence of root rot will lead to the buildup of zoospore inoculum in the soil that may be splashed by rain or irrigation onto low-hanging fruit, causing brown rot. Vice versa, infected fruit that fall to the ground will increase the amount of inoculum in the soil. Therefore, management of *Phytophthora* diseases should be done in an integrated approach, including cultural practices (e.g., tree skirting or not harvesting from the lower tree canopy, irrigation management, use of *Phytophthora*-tolerant rootstocks), and pre- and postharvest fungicide applications. Few fungicides are currently registered in the United States for the control of *Phytophthora* diseases of citrus. Copper products and phosphonates (e.g., fosetyl-Al

and potassium phosphite) are used in fruit and foliar applications to manage brown rot and gummosis, whereas phosphonate and phenylamide (e.g., metalaxyl and mefenoxam) compounds are applied to the soil to reduce root rot. Potassium phosphite was registered in 2013 as the first postharvest fungicide to manage brown rot (Adaskaveg and Förster 2014; Adaskaveg et al. 2015b). The limited number of fungicides available has led to their overuse and subsequent resistance development. Resistance to the phenylamide class of fungicides has been reported in Oomycota pathogens of numerous crops (Gisi and Sierotzki 2015), including citrus, where resistant populations of *P. nicotianae* are established in Florida nurseries (Timmer et al. 1998). Resistance was recently also reported for potassium phosphite in isolates of *P. citrophthora* and *P. syringae* collected from California citrus orchards, and brown rot caused by these isolates could not be controlled using registered rates of potassium phosphite (Adaskaveg et al. 2017; Förster et al. 2016).

New fungicides are becoming available to manage *Phytophthora* diseases of citrus with the pending registrations of four compounds with modes of action different from those of registered fungicides (Blum et al. 2010; Jiang et al. 2015; Pasteris et al. 2016): the thiazole carboxamide ethaboxam, the benzamide fluopicolide, the carboxylic acid amide mandipropamid, and the piperidinyl thiazole isoxazoline oxathiapiprolin. These new chemistries each have unique and different modes of action. Mandipropamid is suspected to target a cellulose synthase gene vital to cell wall biosynthesis (Blum et al. 2010). Ethaboxam inhibits mitosis and cell division by disrupting microtubules (Uchida et al. 2005). Fluopicolide delocalizes spectrin-like proteins, thereby destabilizing plasma membrane formation (Toquin et al. 2010). Oxathiapiprolin inhibits oxysterol-binding proteins affecting all stages in the asexual life cycle (Cohen 2015).

The introduction of four new fungicides with unique modes of action provides an opportunity for designing new effective disease control programs and implementing resistance management strategies that slow the development and spread of resistance while extending the lifespan of existing treatments. To provide a reference for future resistance monitoring and to possibly use the new fungicides against the most sensitive life stages of *Phytophthora* spp., we determined the in vitro toxicities of these compounds. Thus, the objectives of this study were to evaluate in vitro sensitivities of mycelial growth of *P. citrophthora*, *P. syringae*, *P. nicotianae*, and *P. hibernalis* to ethaboxam, fluopicolide, mandipropamid, and oxathiapiprolin; compare the fungicides with mefenoxam; and assess the toxicity of these

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fungicides to other selected stages in the life cycles of *Phytophthora* spp. on citrus in California.

Materials and Methods

Isolates of *Phytophthora* and fungicides used. Isolates of *Phytophthora* were obtained from infected root, fruit, and soil samples from major citrus-growing regions in California. The majority was collected between 2012 and 2016 as part of a survey study (Hao et al. 2018) (Table 1). For evaluation of fungicide toxicity in this study, 62 isolates of *P. citrophthora* (from orange fruit, soil, and roots), 71 isolates of *P. syringae* (from orange fruit), and 31 isolates of *P. nicotianae* (from citrus soil and roots) were used. Only two isolates of *P. hibernalis* (both from grapefruit) were available. Cultures of working stocks were maintained on clarified 10% V8C agar (V8C) (Ribeiro 1978) in the dark at 25°C for *P. citrophthora* and *P. nicotianae*, 20°C for *P. syringae*, and 18°C for *P. hibernalis*. Long-term preservation was in liquid nitrogen. Fungicides tested were ethaboxam (Intego; Valent USA, Walnut Creek, CA), fluopicolide (Presidio; Valent USA), mandipropamid (Revus; Syngenta Crop Protection, Greensboro, NC), oxathiapiprolin (Orondis; Syngenta Crop Protection), and mefenoxam (Ridomil Gold; Syngenta Crop Protection). Aqueous suspensions of the fungicides were used in all experiments.

In vitro toxicities to mycelial growth. For determining sensitivities of mycelial growth, the spiral gradient dilution (SGD) method (Förster et al. 2004) was used. First, isolates were grown on sterile hydrophilic cellophane strips (5.5 by 0.5 cm; du Pont de Nemours, Wilmington, DE) that were placed onto 10% V8C agar. Mycelium-covered agar strips of 1-week-old cultures of *P. nicotianae* or *P. citrophthora* or 2-week-old cultures of *P. syringae* or *P. hibernalis* were placed between the cellophane strips. Plates were then incubated in the dark at the respective temperature for each species (see above) until mycelia had grown evenly over the cellophane strips (approximately 1 to 2 weeks). For the SGD assay, aqueous stock solutions of ethaboxam (10, 50, 5, or 5 µg/ml for *P. nicotianae*, *P. citrophthora*, *P. syringae*, or *P. hibernalis*, respectively), fluopicolide (100 µg/ml), mandipropamid (10 µg/ml), oxathiapiprolin (5 µg/ml), or mefenoxam (50 µg/ml for *P. nicotianae*, *P. citrophthora*, *P. hibernalis* or 15 µg/ml for *P. syringae*) were radially applied to 15-cm 10% V8C agar plates using a spiral plater (Autoplate 4000; Spiral Biotech, Norwood, MA) in the exponential deposition mode. Sterile water was applied to control plates. After 2 h, a center core of agar approximately 35 mm in diameter was removed and, in total, eight mycelium-covered cellophane strips were placed radially and evenly spaced onto the agar surface. For each isolate, duplicate strips were placed on the opposite sides of the plate, with a total of four duplicated isolates per plate. Plates were then incubated for 2 days at 25°C for *P. citrophthora* and *P. nicotianae*, 4 days at 20°C for *P. syringae*, or 18°C for *P. hibernalis*. The location on the plate where mycelial growth was inhibited by 50% as compared with the water control was determined, and local fungicide concentrations (i.e., 50% effective concentration [EC₅₀] values) were calculated using the SGE software (Spiral Biotech). Each isolate was evaluated in two experiments for each fungicide.

In vitro toxicities to sporangial production. Four isolates of *P. citrophthora* (i.e., 2440, 4569, 4872, and 4873) were tested using a slightly modified procedure that was described previously (Farhi et al. 1981). Isolates were grown for 3 days at 25°C on 10% V8C agar, four 5-mm-diameter plugs from the edge of the colony were transferred mycelium side down to a 6-cm petri dish, and 10% V8C broth was added without submerging the plugs. Plates were incubated under fluorescent light (2,200 lux; Daylight Full Spectrum, 5,000 K, 40 W; Osram Sylvania, Danvers, MA) for 24 h at 25°C, the broth was removed, and mycelial mats were rinsed three times with sterile distilled water. Subsequently, 2 ml of sterile distilled water (control) or selected concentrations of fungicides were added to each plate and plates were incubated under fluorescent light for an additional 24 h. The presence of sporangia developing from new mycelial growth along the entire edge of each mycelial mat was evaluated microscopically at ×100 final magnification. Initially, a range of concentrations for each fungicide (ethaboxam at 0.1 to 100 µg/ml,

fluopicolide at 0.01 to 10 µg/ml, mandipropamid at 0.001 to 1 µg/ml, oxathiapiprolin at 0.0001 to 0.1 µg/ml, and mefenoxam at 1 to 1000 µg/ml, with each range in 10-fold dilutions) was used to determine the lowest concentration that inhibited sporangia formation by ≥90% (EC_{≥90} value) as compared with the water control. The final concentration for ≥90% inhibition was validated in two repeated experiments using four replicates for each isolate and fungicide concentration.

In vitro toxicities to zoospore cyst germination. Four isolates of *P. citrophthora* (i.e., 4867, 4569, 4572, and 4873) were grown as described above to produce sporangia. To induce zoospore release, plates were incubated at 4°C for 20 min and at 25°C for another 20 min. Zoospore suspensions were collected in sterile 50-ml tubes, and tubes were vortexed for 90 s to induce encystment. Fungicide-amended SGD plates were prepared as described above using stock concentrations of ethaboxam, fluopicolide, mandipropamid, oxathiapiprolin, and mefenoxam of 5,000, 5,000, 100, 10, and 5,000 µg/ml, respectively. Cyst suspensions (10 µl of 5 × 10⁴ cysts/ml) were streaked radially across the fungicide gradient from the edge to the center of the plate with a sterilized plastic pellet pestle (Kimble, Rockwood, TN). For each isolate, duplicate streaks were placed on opposite sides of the plate. Plates were incubated at 25°C for 6 h, and cyst germ tubes were observed under a compound microscope at ×100 magnification. A cyst was considered germinated when the length of the germ tube was at least twice as long as the diameter of the cyst. The location on the plate where cyst germination was reduced by 50% as compared with the control was determined, and EC₅₀ values were calculated using the SGE program as described above. This experiment was done three times.

In vitro toxicities to chlamydospore formation. Isolates of *P. nicotianae* (i.e., 2348, 2351, 2355, and 2390) were grown for 3 days on 10% V8C agar at 25°C. Five 5-mm-diameter plugs from the edge of the colony were transferred into sterile 50-ml plastic tubes containing 10 ml of 10% V8C broth. The tubes were incubated horizontally in the dark for 7 days at 20°C. Subsequently, 40 ml of water (control) or an aqueous fungicide solution was added to each tube. There were four replications for each fungicide concentration and isolate. The tubes were incubated under the same conditions as indicated above and, every 3 days, they were inverted three times to optimize exposure to the fungicides. After 3 weeks, the broth was carefully decanted to 5 ml, and the mycelial mats in the remaining broth were blended for 90 s at setting 1 using a Powergen 700 homogenizer (Fisher Scientific, Pittsburgh, PA) equipped with a 20-mm-diameter generator probe. Chlamydospores in each sample were enumerated microscopically at a final magnification of ×40 using a

Table 1. Isolates of *Phytophthora citrophthora*, *P. nicotianae*, and *P. syringae* used for in vitro sensitivity studies

Species	Number of isolates	Years collected	County of origin ^z	Source
<i>P. citrophthora</i>	41	2012-2014	Fresno	Orange, mandarin
	19	2014	Tulare	Orange
	1	Unknown	Ventura	Unknown
	1	Unknown	Unavailable	Orange
<i>P. syringae</i>	52	2012-2014	Fresno	Orange, lemon
	19	2013-2014	Tulare	Orange
<i>P. nicotianae</i>	10	2014	Fresno	Rhizosphere
	1	2001	Riverside	Rhizosphere
	4	pre-1990	San Bernardino	Rhizosphere
	1	1974	San Diego	Rhizosphere
	5	2014	Tulare	Rhizosphere
	1	Unknown	Ventura	Rhizosphere
	9	1980-1990	Unavailable	Rhizosphere

^z Unavailable indicates county location unknown; all isolates were recovered from either central or southern California.

hemacytometer. For each replicate, 10 readings were obtained that were averaged. To obtain EC₅₀ values for inhibition of chlamydospore formation for each fungicide, a range of concentrations (0.1 to 10 µg/ml for ethaboxam, 0.01 to 10 µg/ml for fluopicolide, 0.002 to 2 µg/ml for mandipropamid, 0.002 to 2 µg/ml for oxathiapiprolin, and 0.02 to 20 µg/ml for mefenoxam, each in 10-fold dilutions) was first evaluated, and the final concentration for ≥90% inhibition was validated in two repeated experiments using four replicates for each isolate and fungicide concentration.

In vitro toxicities to oospore formation. Two *P. nicotianae* isolates of opposite mating types (isolate 2358 = A1 and isolate 2350 = A2) were grown as described above to produce zoospores. Zoospore suspensions (250 µl of each isolate at 1.3×10^5 zoospores/ml/plate) were uniformly spread across 6-cm petri plates containing 5 ml of 10% V8C agar. The agar surface was air dried, and plates were incubated at 25°C for 20 h. Fungicide solutions (5 ml) were then added to the plates (two replicates for each concentration). Plates were incubated for 1 h to allow the fungicide to diffuse into the agar. The solutions were drained, another 5 ml of the same fungicide solution was added, and plates were incubated for an additional hour. The plates were drained again and incubated at 25°C in the laboratory with a 12-h photoperiod for 7 days, and oospore formation was assessed. For this, three agar plugs from each plate were excised, placed on a glass slide, and microscopically examined at ×40 final magnification to determine the number of oospores within a field of view (0.44 mm in diameter). Initial fungicide concentrations used were within a wide range, similar as for evaluation of sporangium production above, and final concentrations were 0.002, 0.02, 0.1, and 0.2 µg/ml for ethaboxam; 0.1, 0.2, and 0.4 µg/ml for fluopicolide; 0.004, 0.02, and 0.04 µg/ml for mandipropamid; 0.00004, 0.0002, 0.0004, and 0.0006 µg/ml for oxathiapiprolin; and 0.5, 1, 20, and 40 µg/ml for mefenoxam. These final concentrations were evaluated two times.

Statistical analysis of data. EC₅₀ values for mycelial growth inhibition by each fungicide were analyzed using frequency histograms. Standard deviations for each isolate were calculated from log₁₀-transformed EC₅₀ values and used to calculate EC₅₀ category bin width values as described by Scott (Scott 1979) using the equation $h = 3.49sn^{-1/3}$, where h is the bin width of each EC₅₀ category group, s is an estimate of the standard deviation, and n is the number of isolates that were tested with each fungicide. Using the calculated bin widths, the number of isolates within each bin was determined, and bins were graphed in frequency histograms over the EC₅₀ value range obtained.

For comparisons of the effect of the five fungicides on sporangial formation of *P. citrophthora*, data were converted to percent reduction as compared with the untreated control and arcsin transformed, variances were analyzed using Bartlett's test of homogeneity, and homogeneous data sets were combined. Mean values and standard errors were calculated for each fungicide concentration and graphed against percent reduction in sporangial formation.

For comparison of the toxicity of the five fungicides against selected *Phytophthora* life stages, a subset of four isolates of *P. citrophthora* and *P. nicotianae* was evaluated, except for oospore formation, where only one cross was used. Data for EC values were log₁₀ transformed,

variances were analyzed using Bartlett's test of homogeneity, and homogeneous data sets were combined. Statistical comparisons were made among fungicides and species using a two-way factorial design. A one-way classification of data was used for comparing fungicides and life stages for each species following analysis of variance or general linear model and least significant difference mean separation procedures (SAS ver. 9.4; SAS Institute, Cary, NC).

Results

In vitro toxicities to mycelial growth of *Phytophthora* spp. from citrus. The four *Phytophthora* spp. evaluated all were highly sensitive to ethaboxam, fluopicolide, mandipropamid, and oxathiapiprolin. Oxathiapiprolin had the lowest EC₅₀ values against mycelial growth, with ranges and mean values of 0.0002 to 0.0015 and 0.0003 µg/ml for *P. citrophthora*, 0.00006 to 0.0003 and 0.0001 µg/ml for *P. syringae*, 0.0003 to 0.0010 and 0.0005 µg/ml for *P. nicotianae*, and <0.0003 µg/ml for *P. hibernalis* (Table 2). Sensitivity ranges and mean values for ethaboxam, fluopicolide, and mandipropamid were 0.005 to 0.130 and 0.068 µg/ml, 0.031 to 0.087 and 0.040 µg/ml, and 0.002 to 0.005 and 0.004 µg/ml for *P. citrophthora*; 0.001 to 0.038 and 0.005 µg/ml, 0.020 to 0.461 and 0.045 µg/ml, and 0.002 to 0.011 and 0.003 µg/ml for *P. syringae*; 0.001 to 0.050 and 0.016 µg/ml, 0.039 to 0.095 and 0.057 µg/ml, and 0.002 to 0.008 and 0.005 µg/ml for *P. nicotianae*; and 0.026 to 0.034 and 0.030 µg/ml, 0.017 to 0.018 and 0.018 µg/ml, and 0.004 to 0.005 and 0.005 µg/ml, respectively, for the two isolates of *P. hibernalis* that were available for the study. Thus, ranges of sensitivity values for the new fungicides were often narrow but, for ethaboxam, differences between the lowest and highest values were at least 26-fold for the first three species. A 23-fold range was observed for fluopicolide against *P. syringae*, where an inhibitory concentration of 0.461 µg/ml was determined for one isolate, the highest value for all isolates and fungicides. For comparison, sensitivity ranges and mean values for the registered mefenoxam were 0.013 to 0.150 and 0.039 µg/ml, 0.003 to 0.082 and 0.008 µg/ml, 0.050 to 0.400 and 0.183 µg/ml, and ≤0.001 and ≤0.001 µg/ml for *P. citrophthora*, *P. syringae*, *P. nicotianae*, and *P. hibernalis*, respectively.

Frequency histograms of EC₅₀ values for the five fungicides evaluated mostly showed a unimodal distribution of sensitivities among isolates of each species (Figs. 1, 2, and 3). Furthermore, 58 of 62 isolates of *P. citrophthora* had EC₅₀ values within a narrow range for mandipropamid and were placed into a single EC₅₀ category bin (Fig. 1C).

In vitro toxicities to sporangial production of *P. citrophthora*. In nonamended control plates, the average number of sporangia around the edge of a fungal colony in two experiments was 480, 1,761, 1,090, and 533 for isolates 4872, 4569, 4873, and 2440, respectively. Fungicides varied widely in their activity to inhibit sporangial production of *P. citrophthora* (Table 3). Oxathiapiprolin and mandipropamid were statistically ($P < 0.001$) the most active against this life stage, and mean concentrations to inhibit sporangial formation of four isolates by ≥90% were 0.005 and 0.026 µg/ml, respectively. Values for ethaboxam and fluopicolide were 0.1 and 0.28 µg/ml, respectively, whereas, for mefenoxam, mycelial mats had to be incubated in an average of 55 µg/ml to obtain a 90% reduction in sporangia. At the high concentrations of each fungicide evaluated, >90% reduction was obtained. Still,

Table 2. In vitro sensitivity ranges and mean 50% effective concentration (EC₅₀) values for inhibition of mycelial growth of *Phytophthora syringae*, *P. citrophthora*, *P. nicotianae*, and *P. hibernalis* for four new Oomycota-specific fungicides as compared with mefenoxam

Fungicide	Range of EC ₅₀ values (µg/ml)/mean ^z			
	<i>P. citrophthora</i>	<i>P. syringae</i>	<i>P. nicotianae</i>	<i>P. hibernalis</i>
Ethaboxam	0.005–0.130/0.068	0.001–0.038/0.005	0.001–0.050/0.016	0.026–0.034/0.030
Fluopicolide	0.031–0.087/0.040	0.020–0.461/0.045	0.039–0.095/0.057	0.017–0.018/0.018
Mandipropamid	0.002–0.005/0.004	0.002–0.011/0.003	0.002–0.008/0.005	0.004–0.005/0.005
Oxathiapiprolin	0.0002–0.0015/0.0003	0.00006–0.0003/0.0001	0.0003–0.0010/0.0005	<0.0003
Mefenoxam	0.013–0.150/0.039	0.003–0.082/0.008	0.050–0.400/0.183	≤0.001

^z EC₅₀ values were determined using the spiral gradient dilution method for 62 isolates of *P. citrophthora*, 71 isolates of *P. syringae*, 31 isolates of *P. nicotianae*, and 2 isolates of *P. hibernalis*.

lower concentrations of each fungicide also resulted in substantial reductions (>60%) of sporangium formation (Fig. 4).

In vitro toxicities to zoospore cyst germination of *P. citrophthora*.

On nonamended control plates, $\geq 90\%$ of the plated zoospore cysts of each of the four isolates germinated within 4 h of incubation, and plates were evaluated after 6 h. EC_{50} values for inhibition of germination again varied widely among the five fungicides evaluated. Oxathiapiprolin and mandipropamid again had the lowest values compared with fluopicolide, mefenoxam, or ethaboxam, and ranges for 50% inhibitory values and mean values for the four isolates evaluated were 0.007 to 0.010 $\mu\text{g/ml}$ and 0.008 $\mu\text{g/ml}$ and 0.014 to 0.046 $\mu\text{g/ml}$ and 0.026 $\mu\text{g/ml}$, respectively (Table 3). Oxathiapiprolin was significantly ($P < 0.0001$) more effective than mandipropamid (Table 3). In contrast, cyst germination was not inhibited by ethaboxam, fluopicolide, and mefenoxam at 40, 60, and 64 $\mu\text{g/ml}$, respectively, the highest concentrations evaluated for these fungicides.

In vitro toxicities to chlamydospore formation of *P. nicotianae*.

Average chlamydospore concentrations in blended culture suspensions of untreated controls ranged between 7.3×10^3 and 19.6×10^4 chlamydospores/ml for the four isolates used for this study. The five fungicides evaluated were all effective in inhibiting chlamydospore formation (Table 4). Oxathiapiprolin was significantly ($P < 0.001$)

the most effective compound, inhibiting chlamydospore formation of the four isolates by $\geq 90\%$ as compared with the controls using a concentration of 0.002 $\mu\text{g/ml}$. Inhibitory concentrations for mandipropamid and mefenoxam were similar and not significantly different from each other, with $\geq 90\%$ inhibition using each fungicide at 0.2 $\mu\text{g/ml}$. Inhibitory concentrations for ethaboxam and fluopicolide at 1 $\mu\text{g/ml}$ were significantly higher than the other fungicides evaluated.

In vitro toxicities to oospore formation of *P. nicotianae*. Of the five fungicides examined, all but mefenoxam were effective in inhibiting oospore formation at low concentrations (Table 4). A reduction of $\geq 90\%$ ($EC_{\geq 90}$) in oospore formation in a pairing of opposite mating types of *P. nicotianae* was obtained by oxathiapiprolin at 0.0004 $\mu\text{g/ml}$, mandipropamid at 0.02 $\mu\text{g/ml}$, ethaboxam at 0.1 $\mu\text{g/ml}$, or fluopicolide at 0.4 $\mu\text{g/ml}$. Oxathiapiprolin was significantly ($P < 0.0037$) more effective than the other fungicides, whereas ethaboxam, fluopicolide, and mandipropamid were statistically intermediate. Mefenoxam was found to be relatively ineffective, and concentrations of 40 $\mu\text{g/ml}$ reduced oospore production by only 62%.

Comparison of toxicity against different life stages. A significant interaction ($P < 0.016$) was observed among fungicides and the species evaluated (i.e., *P. citrophthora* and *P. nicotianae*) when

Fig. 1. Frequency histograms of effective concentrations to inhibit mycelial growth of 62 isolates of *Phytophthora citrophthora* by 50% (EC_{50} values) for A, ethaboxam, B, fluopicolide, C, mandipropamid, D, oxathiapiprolin, and E, mefenoxam, as determined by the spiral gradient dilution method. Bar height represents the number of isolates within each bin, and bin widths were calculated using Scott's method (Scott 1979).

a statistical comparison of EC_{50} values for mycelial growth was performed. Thus, the toxicity of the fungicides to mycelial growth was done for each species separately. For *P. citrophthora*, oxathiapiprolin had significantly ($P < 0.0001$) the lowest EC_{50} value ($0.0008 \mu\text{g/ml}$) of the fungicides evaluated, followed by mandipropamid (Table 3). Ethaboxam and fluopicolide had similar EC_{50} values and were not significantly different from each other, whereas mefenoxam was least effective, with an EC_{50} value of $0.107 \mu\text{g/ml}$. For *P. nicotianae*, EC_{50} values of all the fungicides were all significantly ($P < 0.0001$) different from each other. Still, oxathiapiprolin had significantly ($P < 0.0001$) the lowest value, followed by mandipropamid, fluopicolide, ethaboxam, and mefenoxam (Table 4).

A comparison of life stage sensitivity to each fungicide was performed. For *P. citrophthora*, mycelial growth and sporangial formation were significantly more sensitive to ethaboxam ($P < 0.0001$) and fluopicolide ($P < 0.0001$) than cyst germination, whereas mycelial growth was more sensitive ($P < 0.0001$) to mefenoxam than sporangium formation or cyst germination. There was no significant difference in sensitivity of the life stages evaluated for mandipropamid ($P > 0.119$) or oxathiapiprolin ($P > 0.127$) (Table 3). For *P. nicotianae*, mycelial growth and oospore formation were significantly ($P < 0.0001$) more sensitive to ethaboxam than chlamydospore formation, whereas, mycelial growth was more sensitive to fluopicolide ($P < 0.0001$) and mandipropamid ($P < 0.0001$) than either chlamydospore

or oospore formation. Oospore formation was more sensitive to oxathiapiprolin ($P < 0.0004$) than either mycelial growth or chlamydospore formation. In general, mycelial growth was more sensitive to mefenoxam ($P < 0.0002$) than the other life stages of both pathogens (Tables 3 and 4).

Discussion

The ubiquitous occurrence of *Phytophthora* spp. in citrus-growing areas worldwide often requires integrated management strategies to successfully establish and maintain orchards in order to produce a high-quality crop. In California, a high level of disease control is also required to minimize detection of any fruit with brown rot upon arrival in export markets where *P. syringae* and *P. hibernalis* are quarantine pathogens (Adaskaveg et al. 2015a). Quarantine laws prohibit the movement of diseased commodities containing pathogens not present in the import country. The use of cultural practices such as irrigation methods that minimize wetting of fruit, removal of lower branches, and harvesting above a selected height to exclude fruit exposed to splashing water and soil contamination provide some level of brown rot management. Still, the use of fungicides has become an integral part of citrus production in preventing fruit from developing brown rot during transit to the port of arrival of the export market. This, however, is hampered by the current limited number of treatments available and the development of resistance to the phenylamides and,

Fig. 2. Frequency histograms of effective concentrations to inhibit mycelial growth of 71 isolates of *Phytophthora syringae* by 50% (EC_{50} values) for **A**, ethaboxam, **B**, fluopicolide, **C**, mandipropamid, **D**, oxathiapiprolin, and **E**, mefenoxam, as determined by the spiral gradient dilution method. Bar height represents the number of isolates within each bin, and bin widths were calculated using Scott's method (Scott 1979).

more recently, phosphonates in *Phytophthora* populations (Adaskaveg et al. 2017; Förster et al. 2016). The pending registration of the new active ingredients ethaboxam, fluopicolide, mandipropamid, and oxathiapiprolin will be an important step to increase treatment options

for improved disease management and to reduce the risk of resistance development due to overuse of any single mode of action. The Fungicide Resistance Action Committee considers the resistance risk of ethaboxam and mandipropamid as low to medium and of oxathiapiprolin as

Fig. 3. Frequency histograms of effective concentrations to inhibit mycelial growth of 31 isolates of *Phytophthora nicotianae* by 50% (EC₅₀ values) for **A**, ethaboxam, **B**, fluopicolide, **C**, mandipropamid, **D**, oxathiapiprolin, and **E**, mefenoxam, as determined by the spiral gradient dilution method. Bar height represents the number of isolates within each bin, and bin widths were calculated using Scott's method (Scott 1979).

Table 3. Comparative toxicity of ethaboxam, fluopicolide, mandipropamid, oxathiapiprolin, and mefenoxam against selected life stages of *Phytophthora citrophthora* from citrus in California^y

Pathogen, fungicide	EC ₅₀ mycelial growth (µg/ml)			EC _{≥90} sporangium formation (µg/ml)			EC ₅₀ cyst germination (µg/ml)		
	Range	Mean	LSD	Range	Mean	LSD	Range	Mean	LSD
<i>P. citrophthora</i>									
Ethaboxam	0.026–0.137	0.064	B, b	0.1	0.1	B, b	>40 ^z	>40	A, a
Fluopicolide	0.047–0.073	0.061	B, b	0.01–1.00	0.28	BC, b	>60 ^z	>60	A, a
Mandipropamid	0.004–0.005	0.005	C, a	0.001–0.100	0.026	CD, a	0.014–0.046	0.026	B, a
Oxathiapiprolin	0.0005–0.0015	0.0008	D, a	0.0001–0.010	0.005	D, a	0.007–0.010	0.008	C, a
Mefenoxam	0.09–0.122	0.107	A, b	10–100	55.0	A, a	>64 ^z	>64	A, a

^y Values represent the range and mean of inhibitory concentrations for the same four isolates evaluated in two repeated experiments. A single value indicates that the four isolates had the same inhibitory value. EC₅₀ = 50% effective concentration and EC_{≥90} = the concentration that inhibited sporangia formation by ≥90%. Mean EC values were compared using least significant difference (LSD) mean separation procedures. Values followed by the same uppercase letter within a column or values followed by the same lowercase letter within a row are not significantly different for different life stages ($P > 0.05$).

^z Values represent the highest concentration evaluated, and these were included in the statistical analyses.

medium to high, whereas the risk for fluopicolide is currently not known (FRAC 2017).

This is the first study comparing in vitro toxicities of these new Oomycota fungicides to important stages in the disease cycles of *Phytophthora* spp. from major citrus production areas in California. Mycelial growth responses were evaluated for all isolates included in the study because mycelium is the main somatic structure that is capable of surviving from season to season in infected plant material if climatic conditions allow (Fawcett 1936). Sporangia are readily formed by the four species, and effects of the fungicides on their production were evaluated using *P. citrophthora* as a representative. Sporangia produce zoospores that are the main infective propagules of *Phytophthora* spp., and abundant production is a major cause of disease epidemics during rain and irrigation events, when zoospores are disseminated from the soil or from infected fruit onto low-hanging fruit. Therefore, we also evaluated rate effects of the fungicides to determine the response of sporangia to lower concentrations. Important survival structures of *Phytophthora* spp. are chlamydospores and oospores. The effect of the fungicides on their production was investigated using *P. nicotianae*, where these structures are known to occur and are considered critical for persistence during the colder winter months (Lutz and Menge 1991). Oospores are also produced by the homothallic *P. syringae* and *P. hibernalis*. Previously, large quantities of oospores in fallen apple leaf litter were found in outbreaks of *P. syringae* in apple orchards in the United Kingdom, suggesting the importance of oospores in pathogen survival (Harris 1979). The source of *P. syringae* inoculum in citrus orchards has not been clearly identified but studies on the disease cycle of the pathogen in citrus are ongoing (Hao et al. 2016a, 2018).

Mycelial growth of all 166 isolates evaluated was found to be sensitive to the four new fungicides, EC₅₀ values for each of the fungicides tested were similar for the four species evaluated, and, thus, no naturally occurring resistance was detected. For *P. citrophthora*, the extremely narrow sensitivity range for mandipropamid as compared with the other species cannot be explained. Because all *Phytophthora* isolates were never previously exposed to these compounds, the sensitivity ranges we are presenting can be referred to as baseline values that can be used as a reference in future monitoring. The phenylamides, however, have been used since the 1980s in California citriculture, and many of the isolates evaluated by us likely were previously exposed to this class of fungicides. Baseline sensitivities in *Phytophthora* populations from citrus were never established for mfenoxam and, therefore, the values presented reflect current sensitivity levels in production orchards.

Sensitivity ranges for oxathiapiprolin were previously established for other Oomycota fungi, including species of *Phytophthora*. For example, EC₅₀ values for oxathiapiprolin for inhibition of mycelial growth of *P. nicotianae* from tobacco (Bittner and Mila 2016; Qu et al. 2016) and *P. capsici* from a variety of hosts (Miao et al.

2016a,b; Olaya et al. 2016) were in a comparable low range, similar to the four species evaluated in our study. Furthermore, sporangial production and zoospore cyst germination of *P. nicotianae* from tobacco were also found to be highly sensitive to oxathiapiprolin (Bittner and Mila 2016), similar to findings for these life stages of *P. citrophthora* in our study. Additionally, we determined a high toxicity for inhibition of chlamydospore and oospore formation of *P. nicotianae*. Thus, oxathiapiprolin represents the most toxic *Phytophthora*-specific fungicide ever evaluated.

In vitro sensitivities were previously also determined by others to mandipropamid, fluopicolide, and ethaboxam for selected species of *Phytophthora*. Sensitivity of mycelial growth, sporangial production, and cyst germination of *P. nicotianae* from tobacco from China (Wang et al. 2013) and Georgia (Qu et al. 2016) were determined for mandipropamid. Toxicity of fluopicolide was evaluated for mycelial growth and zoospore cyst germination of *P. capsici* (Jackson et al. 2010) as well as for mycelial growth, sporangial production, and cyst germination of *P. nicotianae* (Qu et al. 2016); and the toxicity of ethaboxam was established for mycelial growth of *P. infestans* (Kim et al. 2004; Zhang et al. 2005) and *P. capsici* (Kim et al. 2004). These studies all revealed low inhibitory values for these fungicides, comparable with those we determined for species of *Phytophthora* from citrus in respect to mycelial growth and sporangium formation. Cyst germination of *P. citrophthora*, however, was insensitive to ethaboxam (EC₅₀ > 40 µg/ml) and fluopicolide

Fig. 4. Effect of ethaboxam, fluopicolide, mandipropamid, mfenoxam, and oxathiapiprolin on sporangium formation of *Phytophthora citrophthora*. Vertical lines at each data point are standard errors.

Table 4. Comparative toxicity of ethaboxam, fluopicolide, mandipropamid, oxathiapiprolin, and mfenoxam against selected life stages of *Phytophthora nicotianae* from citrus in California^a

Pathogen, fungicide	EC ₅₀ mycelial growth (µg/ml)			EC _{≥90} chlamydospore formation (µg/ml)		EC _{≥90} oospore formation (µg/ml)	
	Range	Mean	LSD	Mean ^y	LSD	Mean ^y	LSD
<i>P. nicotianae</i>							
Ethaboxam	0.001–0.062	0.016	B, b	1.0	A, a	0.1	B, b
Fluopicolide	0.010–0.100	0.052	C, c	1.0	A, a	0.4	B, b
Mandipropamid	0.002–0.007	0.005	D, c	0.2	B, a	0.02	B, b
Oxathiapiprolin	0.0003–0.0011	0.0006	E, b	0.002	C, a	0.0004	C, c
Mefenoxam	0.079–0.399	0.14	A, b	0.2	B, b	>40 ^z	A, a

^a Values represent the range and mean of inhibitory concentrations for the same four isolates evaluated in two repeated experiments. EC₅₀ = 50% effective concentration and EC_{≥90} = the concentration that inhibited chlamydospore or oospore formation by ≥90%. For oospore formation, one pairing of opposite mating types was used. Mean EC values were compared using least significant difference (LSD) mean separation procedures. Values followed by the same uppercase letter within a column or values followed by the same lowercase letter within a row are not significantly different for different life stages ($P > 0.05$).

^y The four isolates evaluated for chlamydospore formation and oospore formation in the one pairing of *P. nicotianae* had the same EC_{≥90} value for each fungicide evaluated.

^z Values represent the highest concentration evaluated, and these were included in the statistical analyses.

($EC_{50} > 60 \mu\text{g/ml}$). In contrast, mandipropamid was highly toxic against all *Phytophthora* life stages evaluated, similar to oxathiapiprolin. Inhibitory values for the different life stages are difficult to compare because EC_{50} values were determined for mycelial growth and cyst germination, whereas $EC_{\geq 90}$ values were determined for sporangium, chlamydospore, and oospore formation. The latter discriminatory values were chosen because they were more reliably assessed for these structures that are generally not uniformly produced over the colony. Still, we made statistical comparisons of the sensitivity among life stages of *P. citrophthora* and *P. nicotianae* by comparing EC values to better characterize the activity of the fungicide.

Similar to our evaluations, sensitivity ranges to the new fungicides were generally narrow in previous studies with other species of *Phytophthora*. For fluopicolide, however, EC_{50} values for sporangial production of *P. capsici* ranged from 0.3 to 9.0 $\mu\text{g/ml}$ (i.e., a 30-fold difference) (Jackson et al. 2010). In our study, the EC_{50} range for fluopicolide on mycelial growth inhibition of *P. syringae* was 0.02 to 0.461 $\mu\text{g/ml}$ (i.e., a 23-fold difference) and for ethaboxam was 0.005 to 0.130 (i.e., a 26-fold difference) for *P. citrophthora*. These were the widest ranges of sensitivity values for the *Phytophthora* spp.–fungicide interactions that we evaluated. They possibly indicate a potential for selecting isolates with reduced sensitivity to these fungicides in populations of these *Phytophthora* spp. Because neither fluopicolide or ethaboxam have been used previously in California citrus production, the wide ranges reported cannot be due to any prior exposure to these chemistries. We also determined wider toxicity ranges for mefenoxam for most of the interactions evaluated. This may indicate a shift resulting from many years of field usage. Still, EC_{50} values for mycelial growth inhibition were all $\leq 0.4 \mu\text{g/ml}$ and, therefore, all isolates evaluated can be regarded as sensitive to mefenoxam.

In summary, in this study, we established a high level of in vitro toxicity of ethaboxam, fluopicolide, mandipropamid, and oxathiapiprolin to *Phytophthora* spp. infecting citrus roots, fruit, and tree trunks in California. These fungicides were all highly effective in reducing mycelial growth. Although, for each of the four fungicides, statistical differences were determined in the sensitivity of the life stages evaluated, all sensitivities were $< 1 \mu\text{g/ml}$, except for ethaboxam and fluopicolide for cyst germination and chlamydospore formation. Thus, mandipropamid and oxathiapiprolin were most active in inhibiting sporangial formation, cyst germination, and oospore formation using low concentrations; three life stages that were relatively insensitive to mefenoxam. Oxathiapiprolin was the only compound that was highly toxic at low concentrations ($\leq 0.010 \mu\text{g/ml}$) to all life stages evaluated, including chlamydospore and oospore formation. Registration of these fungicides has the promise to provide a new level of efficacy in managing *Phytophthora* diseases of citrus and other crops. For citrus, this was already demonstrated in field studies, where the incidence of root rot and soil population sizes of *P. nicotianae* were reduced to very low levels and with a higher efficacy as compared with mefenoxam (Hao et al. 2016b).

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