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Evaluation and Characterization of New Fungicides for the Management of Phytophthora Diseases of Citrus in California

A Dissertation submitted in partial satisfaction
of the requirements for the degree of

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in

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by

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Chapter I in its entirety is reprinted with permission as it appears in Gray, M.A., Hao, W., Förster, H., Adaskaveg, J.E. 2018. Baseline Sensitivities of New Fungicides and Their Toxicity to Selected Life Stages of *Phytophthora* Species from Citrus in California. Plant Disease. 102:737-742. Chapter II in its entirety is also reprinted with permission as it appears in Hao, W., Gray, M.A., Förster, H., Adaskaveg, J.E. 2018. Evaluation of New Oomycota Fungicides for The Management of Phytophthora Root Rot of Citrus in California. Plant Disease. Accepted for publication. Doi: https://doi.org/10.1094/PDIS-07-18-1152-RE. Financial support for Chapter I, II, and III was provided by the California Research Board (CRB), Syngenta Crop Protection, Valent USA and Luxembourg Industries Ltd.. Special thanks to these later cooperators for the kind donation of fungicides and analytical grade samples used in Chapters I, II, and III. Citrus plants used in Chapter II were kindly donated by third party nurseries.

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DEDICATION

This dissertation is dedicated with love to my late grandmother Ruth Verhulst Gray, to whom I will always be grateful for instilling in me an appreciation for hard work, patience, and fortitude.

ABSTRACT OF THE DISSERTATION

Evaluation and Characterization of New Fungicides for the Management of Phytophthora Diseases of Citrus in California

by

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University of California, Riverside, March 2019
Dr. James E. Adaskaveg, Chairperson

Phytophthora nicotianae (P. parasitica), *P. citrophthora,* and *P. syringae*, are some of the most destructive *Phytophthora* species known to California citrus production, causing crop losses and in some instances, quarantine trade restrictions. The currently limited number of fungicides for the control of Phytophthora root rot and brown rot outbreaks has led to the over-use of available treatments and resulted in resistance development. In response, we sought to determine the suitability of four new fungicides (i.e. ethaboxam, fluopicolide, mandipropamid, and oxathiapiprolin) being proposed for registration on citrus in California as possible treatments to control Phytophthora diseases of citrus.

The results of this dissertation are presented in studies on 1) the in vitro sensitivity of selected life stages of several species of *Phytophthora* including *P. nicotianae*, *P. citrophthora*, *P. syringae*, and *P. hibernalis* collected from selected citrus production regions in California; 2) greenhouse and field studies on the efficacy of the newly

identified fungicides for managing Phytophthora root rot of citrus; and 3) mobility of oxathiapiprolin and mefenoxam in citrus seedlings after root applications.

In vitro studies established ranges of EC_{50} values for 31, 62, 71, and 2 isolates for *P. nicotianae, P. citrophthora, P. syringae,* and *P. hibernalis*, respectively that can be used as baselines for future comparisons to determine shifts in isolate sensitivity or resistance once the fungicides are used extensively. Most of the isolates were within a narrow range of sensitivity and therefore resistance was not identified to any of the new fungicides. Oxathiapiprolin was the most efficacious fungicide tested in vitro, inhibiting all life stages evaluated including mycelial growth, sporangium and oospore formation, as well as zoospore cyst germination. Ethaboxam, fluopicolide, and mandipropamid were also shown to be highly effective in inhibiting mycelial growth.

In greenhouse and field studies with the new fungicides, efficacy was demonstrated by in reduced root rot incidence and recovery of *P. nicotianae* and *P. citrophthora* from collected soil and root samples. In field trials, positive correlations were also observed in growth trends of treated trees such as increased canopies, trunk diameters, and fruit production following fungicide applications. Fluopicolide and oxathiapiprolin treatments had the greatest effect in promoting the recovery of infected trees and increasing fruit yields.

Studies to determine the systemic nature of oxathiapiprolin in citrus plants were done using bioassay screenings and HPLC-MS/MS analysis of tissue extracts of plants treated with soil applications of oxathiapiprolin or mefenoxam. Oxathiapiprolin and

mefenoxam were detected in leaf, stem, and root tissues indicating that both compounds can be translocated acropetally.

In summary, the information obtained from these studies will help in the registration of new fungicide treatments for managing Phytophthora diseases of citrus, provide four new modes of action with no cross resistance between the different fungicide resistance action committee (FRAC) groups, and ultimately provide a high level of efficacy in the management of Phytophthora root rot and brown rot of citrus in California with a minimal risk of resistance development through the rotational use of these fungicides.

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GENERAL INTRODUCTION

Phytophthora species are Oomycete organisms within the Kingdom Stramenopila that can cause diseases on a wide variety of agricultural crops and non-cultivated plants. Worldwide, several species, including *P. citrophthora, P. syringae, P. nicotianae, P. citricola, P. palmivora,* and *P. hibernalis,* are pathogens of citrus (Erwin and Ribeiro 1996). Within California, many of these including *P. citrophthora, P. syringae, P. parasitica,* and *P. hibernalis* have been recovered from citrus. These species are active at different times of the year with *P. syringae* and *P. hibernalis* present in the cooler seasons, *P. parasitica* during the summer, and *P. citrophthora* mostly causing disease during spring, fall, and winter (Fawcett 1936; Hao 2018). They are all capable of causing brown rot of citrus fruit in the orchard and after harvest in storage or during transit. *P. citrophthora* and *P. parasitica* will also cause root rot and gummosis in the orchard which can make the establishment of new plantings difficult, leading to slow tree decline and reductions in productivity once introduced. The impact of Phytophthora diseases on citrus production can be devastating. Epidemics have occurred as far back as 1863-1870 in Italy in which large numbers of lemon trees were destroyed due to gummosis caused by *Phytophthora citrophthora* and *P. parasitica,* along with additional outbreaks in nearby Greece where most of the lemon trees on the island of Paros were destroyed between 1869 to 1880 (Fawcett 1936). More recently, it was estimated that within California, Phytophthora root rot outbreaks can continue to lead to yield losses of up to 46% if left unmanaged (Menge 1993). The California citrus industry is economically important for both the state and country. Fruit produced in California is primarily

earmarked for fresh consumption, with the state producing roughly 59% of the total citrus grown within the United States, valued at around 2.4 billion dollars (USDA-NASS 2018).

Recently, *P. syringae* and *P. hibernalis* were designated quarantine pathogens in China, an important export country for the California citrus industry, following the detection of brown rot infected fruit shipped from California to Chinese ports. Both species were previously considered of minor importance, but in recent years, *P. syringae* has been commonly found causing brown rot of fruit during the winter harvest season in the major citrus production areas in the Central Valley of California (Hao 2018). This subsequently led to the restriction of the California citrus trade and extensive monetary losses (Adaskaveg and Förster 2014). As of 2016, California citrus exports to China, which is one of the top 15 export countries for California citrus, were valued at $133 million dollars (CDFA 2017), underlining the importance of preventing future trade restrictions to this important market due to phytosanitary issues caused by *Phytophthora* spp.

The root and soil phases in the disease cycle of *Phytophthora* spp. are directly connected with the brown rot phase. Under favorable conditions, mainly wetness, high inoculum levels in the soil will cause root rot which can be especially detrimental in nurseries and in the establishment of new orchards. This is when disease management is most critical. It has been shown that trees grown in soil infested with *P. parasitica* or *P. citrophthora* prior to repotting to larger containers were later less afflicted with dieback or stunted growth when treated with soil applications of mefenoxam and fosetyl-Al than trees that were not treated (Ohr et al., 1986). It is also suggested that to prevent the spread

of *Phytophthora* spp. from nursery stock to previously uninfected orchards, roots of potted trees should be inspected and tested for the presence of *Phytophthora* spp. (Graham et al., 2014). Even after planting healthy nursery stock, the application of fungicides is necessary in part because young, fibrous roots are more susceptible to root rot than older roots and it is suggested that fungicides, together with nematicides or a soil fumigant be used to protect replants during the first two years of growth, especially if the orchard has a history of *Phytophthora* problems (Adaskaveg et. al, 2014b).

The buildup of inoculum during root rot outbreaks (e.g., sporangia and motile zoospores) can quickly grow and during rain or irrigation events. Sporangia or zoospores, sometimes together, may be splashed up to low-hanging fruit and cause outbreaks of brown rot. Brown rot develops as light brown, leathery lesions that expand until the whole fruit turns brown. Diseased fruit have a characteristic pungent odor and at high humidity, the fruit surface is eventually covered by distinct white mycelium. Both the root rot and brown rot phases are damaging, though while fruit brown rot leads to immediate crop loss, root rot outbreaks instead results in a slow decline of infected trees. Trees infected by *Phytophthora* spp. eventually show reduced vigor and production. Systematic dieback of the canopy occurs when uptake of water and nutrients is restricted due to poor root health caused by the decay of feeder roots. Following infection of the roots, the root cortex is eventually degraded, leaving only the inner stele and giving the root system a stringy appearance (Graham and Menge, 2000). This underlines the importance of root rot management in both an integrated brown rot management program

to prevent both the build-up of inoculum in citrus orchards and as a means to maintain a productive orchard.

Currently the control of root rot and brown rot is addressed through integration of cultural practices (e.g., irrigation management, tree skirting) and fungicide applications. Among cultural practices, the usage of resistant rootstocks has long been utilized for the management of Phytophthora root rot and gummosis issues. Trifoliate and hybrid rootstocks such as C-35 and C-32 citrange are tolerant of root rot and gummosis. Other citrange rootstocks such as Carrizo and Troyer citrange are intermediate in their tolerance to root rot but are tolerant of gummosis (Ferguson, 1990; Graham and Menge, 2000; Adaskaveg et al., 2014b). Irrigation management is another important facet to controlling the spread of Phytophthora root rot, brown rot and gummosis. It has previously been shown that irrigation management that avoids over-watering can significantly reduce populations of *Phytophthora* species in the soil by preventing saturated soil conditions that are conducive to sporangia formation, zoospore release, and zoospore motility (Feld and Menge 1990). To promote consistent drainage and reduce the presence of saturated soil, trees can be planted on raised berms and micro-sprinkler irrigation systems installed to replace furrow irrigation which easily oversaturates the soil. Additional in-season and preharvest fungicide applications, however, are often still needed to control root rot, especially in the establishment of new orchards, and to manage brown rot during the rainy winter harvest season. Preharvest applications of copper or phosphonates (e.g., fosetyl-Al, potassium phosphite) are used to manage brown rot, and phosphonate or phenylamide (e.g., metalaxyl, mefenoxam) fungicides are applied to the soil to reduce

root rot. The limited number of chemistries available for controlling Phytophthora diseases and few options for fungicide rotations has led to their overuse and resulted in the development of resistance (Davidse et al. 1981; Förster et al. 2016; Gisi and Sierotzki 2015). Several formulations of the phenylamide fungicides metalaxyl and mefenoxam have been used by growers for many years to control root rot and gummosis since their introduction. It is known that *Phytophthora* species can develop resistance to this fungicide class after repeated exposure (Cohen and Coffey 1986; Timmer et al. 1998). It has also been observed that phenylamide-resistant populations tend to decrease in the absence of selection pressure (i.e., applications of phenylamide fungicides). This suggests that reducing usage of phenylamide-containing products could eventually make this fungicide class effective again when used strategically in a resistance management program (Staub 1991). The new chemistries ethaboxam, fluopicolide, mandipropamid and oxathiapiprolin all possess unique modes of action, all different from one another and from currently registered products (Uchida et al. 2005; Toquin et al. 2006; Blum et al. 2010; Pasteris et al. 2016). The availability of new modes of action will facilitate the implementation of resistance management strategies using fungicide rotations that will minimize the risk of resistance development. As a result of our previous work (Gray et al. 2018; Hao et al. 2018b), oxathiapiprolin has since been federally and state registered for usage on citrus, while the remaining fungicides are still pending registrations for field application use. Once these fungicides are registered, the implementation of new fungicide rotations with existing chemistries will help not only to manage Phytophthora diseases but also slow the development of resistance, maximize the lifespan of available

chemical treatments, and help ensure continued trade with foreign markets by reducing the prevalence of Phytophthora root rot and brown rot in orchards, effectively lowering the disease incidence to below detection levels.

Thus, the objectives of the following studies were to: (i) Identify the efficacy of new fungicides ethaboxam, fluopicolide, mandipropamid and oxathiapiprolin, at inhibiting several different life stages of *Phytophthora* spp. known to cause root rot, and to determine baseline sensitivity references for future resistance monitoring efforts using isolates of several *Phytophthora* spp. collected from throughout California citrus production regions; (ii) evaluate these new compounds in both greenhouse and field settings to verify their efficacy as potential root rot treatments and determine potential growth promotion benefits of the fungicides and (iii) ascertain a better understanding of the properties of oxathiapiprolin in citrus plant systems following application to determine if oxathiapiprolin is capable of systemic translocation for the purposes of refining potential application practices and improve management of Phytophthora diseases common to citrus within California.

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CHAPTER I. 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 EC50 values to inhibit mycelial growth by ethaboxam, fluopicolide, mandipropamid, oxathiapiprolin, and mefenoxam were 0.068, 0.04, 0.004, 0.0003, and 0.039 µg/ml, for 62 isolates of *P. citrophthora*; 0.005, 0.045, 0.003, 0.0001, and 0.008 µg/ml for 71 isolates of *P. syringae*; 0.016, 0.057, 0.005, 0.0005, and 0.183 µg/ml, for 31 isolates of *P. nicotianae*; and 0.030, 0.018, 0.005, <0.0003, and ≤0.001 µg/ml, for two isolates of *P. hibernalis*. Mean values for >90% inhibition of sporangia formation of four isolates of *P. citrophthora* were 0.1, 0.28, 0.026, 0.005, and 55 µg/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 >90% reduction (EC>90) of 0.002 µg/ml, moderately sensitive to mandipropamid (EC>90 = 0.2 µg/ml) and mefenoxam (EC>90 = 0.6 µg/ml), and least sensitive to ethaboxam and fluopicolide (EC>90 = 1 µg/ml). Oospore formation of *P. nicotianae* was inhibited by >90% using 0.0004 µg/ml

oxathiapiprolin, 0.02 µg/ml mandipropamid, 0.1 µg/ml ethaboxam, or 0.4 µg/ml fluopicolide, whereas 62% inhibition was obtained by 40 µg/ml mefenoxam.

INTRODUCTION

Phytophthora spp. are plant pathogens in the Kingdom Stramenopila that cause diseases on a wide variety of cultivated and non-cultivated plants. Numerous species have been associated with citrus crops worldwide (Erwin and Ribeiro 1996), and in California, *P. 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. 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* species 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, potassium phosphite) are used in fruit and foliar applications to manage brown rot and gummosis, whereas phosphonate and phenylamide (e.g., metalaxyl, 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 over-use, and in 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 (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. 2015): the thiazole carboxamide ethaboxam, the benzamide fluopicolide, the carboxylic acid amide (CAA) 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. 2006). 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 to implement 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 and compare to mefenoxam, and assess the toxicity of these fungicides to other selected stages in the life cycles of *Phytophthora* species 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. 2015a, Hao et al. 2018) (Table 1.1). 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% V8 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 250SC; Syngenta Crop Protection, Greensboro, NC), oxathiapiprolin (Orondis; Syngenta Crop Protection), and mefenoxam (Ridomil Gold SL; 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 cm × 0.5 cm; du Pont de Nemours, Wilmington, DE) that were placed onto 10% V8C agar. Mycelium-covered agar strips of one-week-old cultures of *P. nicotianae* or *P. citrophthora* or two-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 one to two 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*; 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 two hours, a center core of agar approximately 35 mm in diameter was removed and a total of 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* or for 4 days at 20˚C for *P. syringae* or at 18˚C for *P. hibernalis.* The location on the plate where mycelial growth was inhibited by 50% as compared to the water control was determined, and local fungicide concentrations (i.e., EC_{50} 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, 4873) were tested using a slightly modified procedure that was described previously (Farih 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, 5,000K, 40W; Osram Sylvania, Danvers, MA) for 14 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 100x final magnification. Initially, a range of concentrations for each fungicide (0.1 to 100 µg/ml ethaboxam, 0.01 to 10 µg/ml fluopicolide, 0.001 to 1 µg/ml mandipropamid, 0.0001 to 0.1 µg/ml oxathiapiprolin, 1 to 1000 µg/ml mefenoxam, with each range in ten-fold dilutions) was used to determine the lowest concentration that inhibited sporangia formation by ≥90% ($EC_{\geq 90}$ value) as compared to the water control. The final concentration for >90% inhibition was validated in two repeated experiments using 4 replicates for each isolate and fungicide concentration.

In vitro toxicities to zoospore cyst germination. Four isolates of *P. citrophthora* (i.e., 4867, 4569, 4572, 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 5000, 5000, 100, 10, and 5000 µg/ml, respectively.

Cyst suspensions (10 µl of 5×10^4 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 100x 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 to the control was determined, and EC_{50} 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, 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 and of 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 three days, they were inverted three times to optimize exposure to the fungicides. After three 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 40x using a hemacytometer. For each

replicate, ten readings were obtained that were averaged. To obtain $EC_{\geq 90}$ 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, 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 4 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 1.3×10^5 zoospores/ml of each isolate per 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, 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 40x 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. EC_{50} values for mycelial growth inhibition by each fungicide were analyzed using frequency histograms. Standard deviations for each isolate were calculated from $\log_{10}$-transformed EC_{50} values and used to calculate EC_{50} category bin width values as described by Scott (Scott 1979) using the following equation:

$$h = 3.49sn^{-1/3}$$

where h is the bin width of each EC_{50} 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 EC_{50} 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 to the untreated control, 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 $\log_{10}$ transformed and 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 ANOVA or GLM and least significant difference mean separation procedures (SAS ver. 9.4, SAS Institute, Cary, NC).

RESULTS

In vitro toxicities to mycelial growth of *Phytophthora* species from citrus. The four *Phytophthora* species evaluated all were highly sensitive to ethaboxam, fluopicolide, mandipropamid, and oxathiapiprolin. Oxathiapiprolin had the lowest EC_{50} values against mycelial growth with ranges and mean values of 0.0002 to 0.0015 µg/ml and 0.0003 µg/ml for *P. citrophthora*, 0.00006 to 0.0003 µg/ml and 0.0001 µg/ml for *P. syringae*, 0.0003 to 0.0010 µg/ml and 0.0005 µg/ml for *P. nicotianae*, and <0.0003 µg/ml for *P. hibernalis* (Table 1.2). 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 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 EC_{50} values for the five fungicides evaluated mostly showed a uni-modal distribution of sensitivities among isolates of each species (Figs. 1.1 to 1.1.3). Furthermore, 58 of 62 isolates of *P. citrophthora* had EC_{50} values within a narrow range for mandipropamid and were placed into a single EC_{50} category bin (Fig. 1.1).

In vitro toxicities to sporangial production of *P. citrophthora*. In non-amended control plates, the average number of sporangia around the edge of a fungal colony in two experiments was 480, 1761, 1090, and 533 for isolates 4872, 4569, 4873, and 2440, respectively. Fungicides varied widely in their activity to inhibit sporangial production of *P. citrophthora* (Table 1.3). 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 µg/ml and 0.026 µg/ml, respectively. Values for ethaboxam and fluopicolide were 0.1 µg/ml 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, lower concentrations of each fungicide also resulted in substantial reductions (>60%) of sporangium formation (Fig. 1.2).

In vitro toxicities to zoospore cyst germination of *P. citrophthora*. On non-amended control plates, >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 to fluopicolide, mefenoxam, or ethaboxam, and ranges for 50% inhibitory values and mean values for the four isolates evaluated were 0.007 to 0.010 µg/ml and 0.008 µg/ml and 0.014 to 0.046 µg/ml and 0.026 µg/ml, respectively (Table 1.3). Oxathiapiprolin was significantly ($P<0.0001$) more effective than mandipropamid (Table 1.3). In contrast, cyst germination was not inhibited by ethaboxam, fluopicolide, and mefenoxam at 40 µg/ml, 60 µg/ml, and 64 µg/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/ml and 19.6×10^4/ml for the four isolates used for this study. The five fungicides evaluated were all effective in inhibiting chlamydospore formation (Table 1.3). Oxathiapiprolin was significantly ($P<0.001$) the most effective compound, inhibiting chlamydospore formation of the four isolates by $\geq$90% as compared to the controls using a concentration of 0.002 µg/ml. Inhibitory concentrations for

mandipropamid and mefenoxam generally were similar but significantly different from each other with ≥90% inhibition obtained using 0.2 µg/ml of each fungicide. Inhibitory concentrations for ethaboxam and fluopicolide at 1 µg/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. A reduction of ≥90% ($EC_{\geq 90}$) in oospore formation in a pairing of opposite mating types of *P. nicotianae* was obtained by 0.0004 µg/ml oxathiapiprolin, 0.02 µg/ml mandipropamid, 0.1 µg/ml ethaboxam, or 0.4 µg/ml fluopicolide. 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 µg/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 (e.g., *P. citrophthora* and *P. nicotianae*) when 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 µg/ml) of the fungicides evaluated followed by mandipropamid (Table 1.3). 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 µg/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 1.3).

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 ($P<0.0001$) more sensitive 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 1.3). 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 (Table 1.3).

DISCUSSION

The ubiquitous occurrence of *Phytophthora* species 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 more recently to the phosphonates in *Phytophthora* spp. 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 (FRAC) considers the resistance risk of ethaboxam and mandipropamid as low to medium and of oxathiapiprolin as 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* species 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* species, 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* species 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).

Mycelial growth of all 166 isolates evaluated was found to be sensitive to the four new fungicides, EC_{50} 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 to

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 mefenoxam and therefore, the values presented reflect current sensitivity levels in production orchards.

Sensitivity ranges for ethaboxam, fluopicolide, mandipropamid, and oxathiapiprolin were previously established for other Oomycota fungi, including species of *Phytophthora*. For example, EC_{50} 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), as 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 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. capcisi* (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 to those we determined for species of *Phytophthora* from citrus in respect to mycelial growth and sporangium formation. Cyst germination of *P. citrophthora*, however, was relatively insensitive to ethaboxam (EC_{50} >40 µg/ml) and fluopicolide (EC_{50} >60 µg/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 to 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 µg/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 µg/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* species-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 µg/ml and therefore, all isolates evaluated can be regarded 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* species 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 µg/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 µg/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 to mefenoxam (Hao et al. 2016b).

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Table 1.1. Isolates of *Phytophthora citrophthora*, *P. nicotianae,* and *P. syringae* used for in vitro sensitivity studies

Species	No. of Isolates	Years collected	County of Origin	Source
P. citrophthora				
	41	2012-2014	Fresno	orange, mandarin
	19	2014	Tulare	orange
	1	unknown	Ventura	unknown
	1	unknown	unavailable[a]	orange
P. syringae				
	52	2012-2014	Fresno	orange, lemon
	19	2013-2014	Tulare	orange
P. nicotianae				
	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

[a] Exact county location unknown, all isolates recovered from either central or southern California.

Table 1.2. In vitro sensitivity ranges and mean EC_{50} values for inhibition of mycelial growth of *Phytophthora syringae, P. citrophthora, P. nicotianae*, and *P. hibernalis* for four new Oomycota-specific fungicides as compared to mefenoxam

	Range of EC_{50} values (µg/ml)/mean[a]			
Fungicide	***P. citrophthora***	***P. syringae***	***P. nicotianae***	***P. hibernalis***
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

[a] EC_{50} 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.*

Table 1.3. Comparative toxicity of ethaboxam, fluopicolide, mandipropamid, oxathiapiprolin, and mefenoxam against selected life stages of *Phytophthora citrophthora*, *P. nicotianae*, and *P. syringae* from citrus in California[a]

		EC_{50} mycelial growth (µg/ml)				$EC_{>90}$ sporangium formation (µg/ml)				EC_{50} cyst germination (µg/ml)			
Pathogen	**Fungicide**	**Range**	**Mean**	**LSD[b]**	**LSD[b]**	**Range**	**Mean**	**LSD**	**LSD**	**Range**	**Mean**	**LSD**	**LSD**
P. citrophthora	Ethaboxam	0.026 - 0.137	0.064	B	b	0.1	0.1	B	b	>40[c]	>40	A	a
	Fluopicolide	0.047 - 0.073	0.061	B	b	0.01 - 1.00	0.28	BC	b	>60[c]	>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[c]	>64	A	a

		EC_{50} mycelial growth (µg/ml)				$EC_{>90}$ chlamydospore formation (µg/ml)			$EC_{>90}$ oospore formation (µg/ml)			
	Fungicide	**Range**	**Mean**	**LSD**	**LSD**	**Mean[d]**	**LSD**	**LSD**	**Mean[d]**	**LSD**	**LSD**
P. nicotianae	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	C	a	0.02	B	b
	Oxathiapiprolin	0.0003 - 0.0011	0.0006	E	b	0.002	D	a	0.0004	C	c
	Mefenoxam	0.079 - 0.399	0.14	A	b	0.2	B	b	>40[c]	A	a

[a] Values represent the range and mean of inhibitory concentrations for the same 4 isolates of each species evaluated in two repeated experiments. A single value indicates that the four isolates had the same inhibitory values. For oospore germination of *P. nicotianae*, one pairing of opposite mating types was used.

[b] Mean EC values were compared using least significant difference (LSD) mean separation procedures. For each pathogen, 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 ($P>0.05$).

[c] Values represent the highest concentration evaluated, and these were included in the statistical analyses.

[d] The four isolates evaluated for chlamydospore formation and oospore formation in the one pairing of *P. nicotianae* had the same $EC_{\geq 90}$ value for each fungicide evaluated.

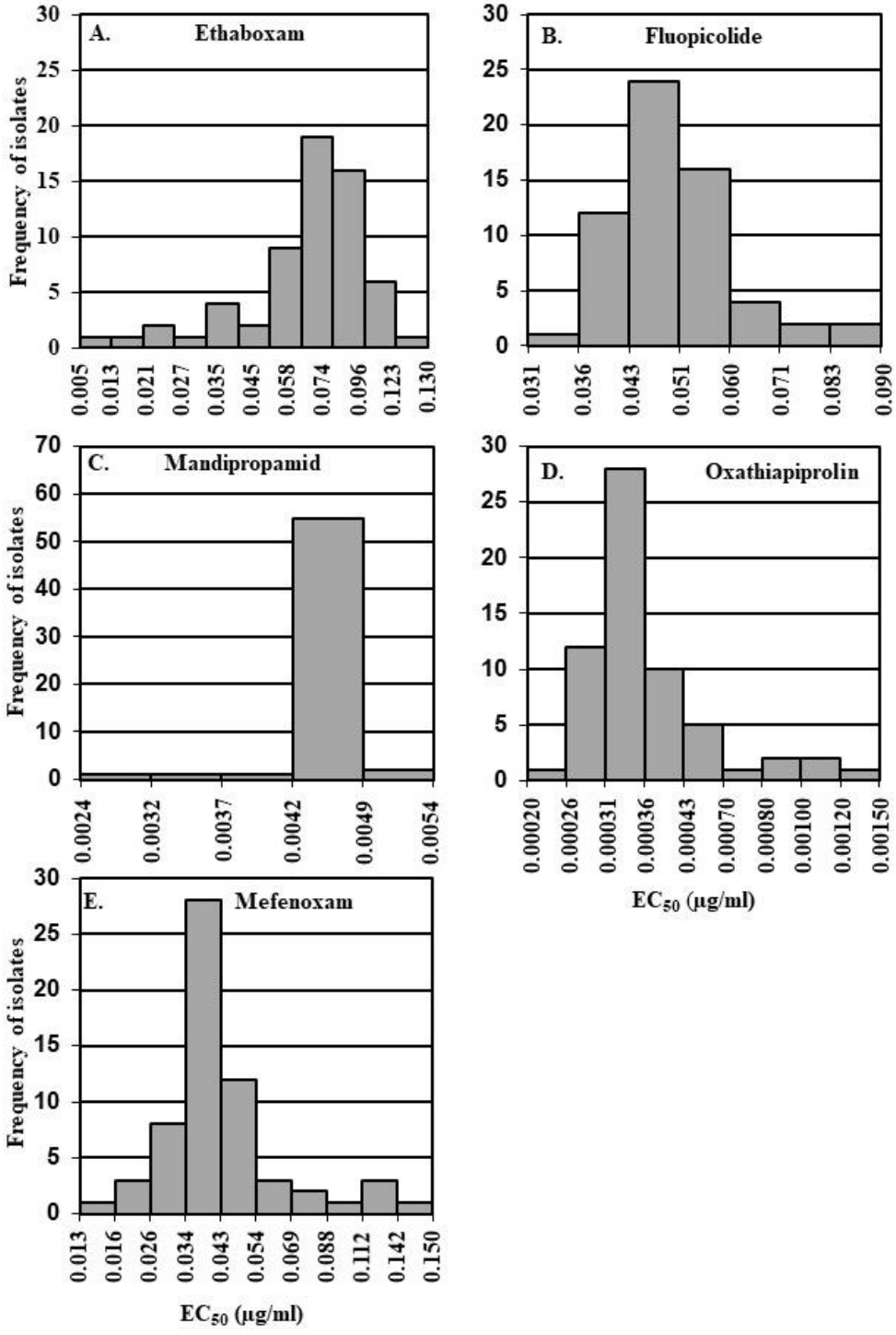

Figure 1.1. Frequency histograms of effective concentrations to inhibit mycelial growth of 62 isolates of *Phytophthora citrophthora* by 50% (EC_{50} values) for **A,** ethaboxam, **B,** fluopicolide, **C,** mandipropamid, **D,** oxathiapiprolin, and **E,** mefenoxam as determined by the spiral gradient dilution method.

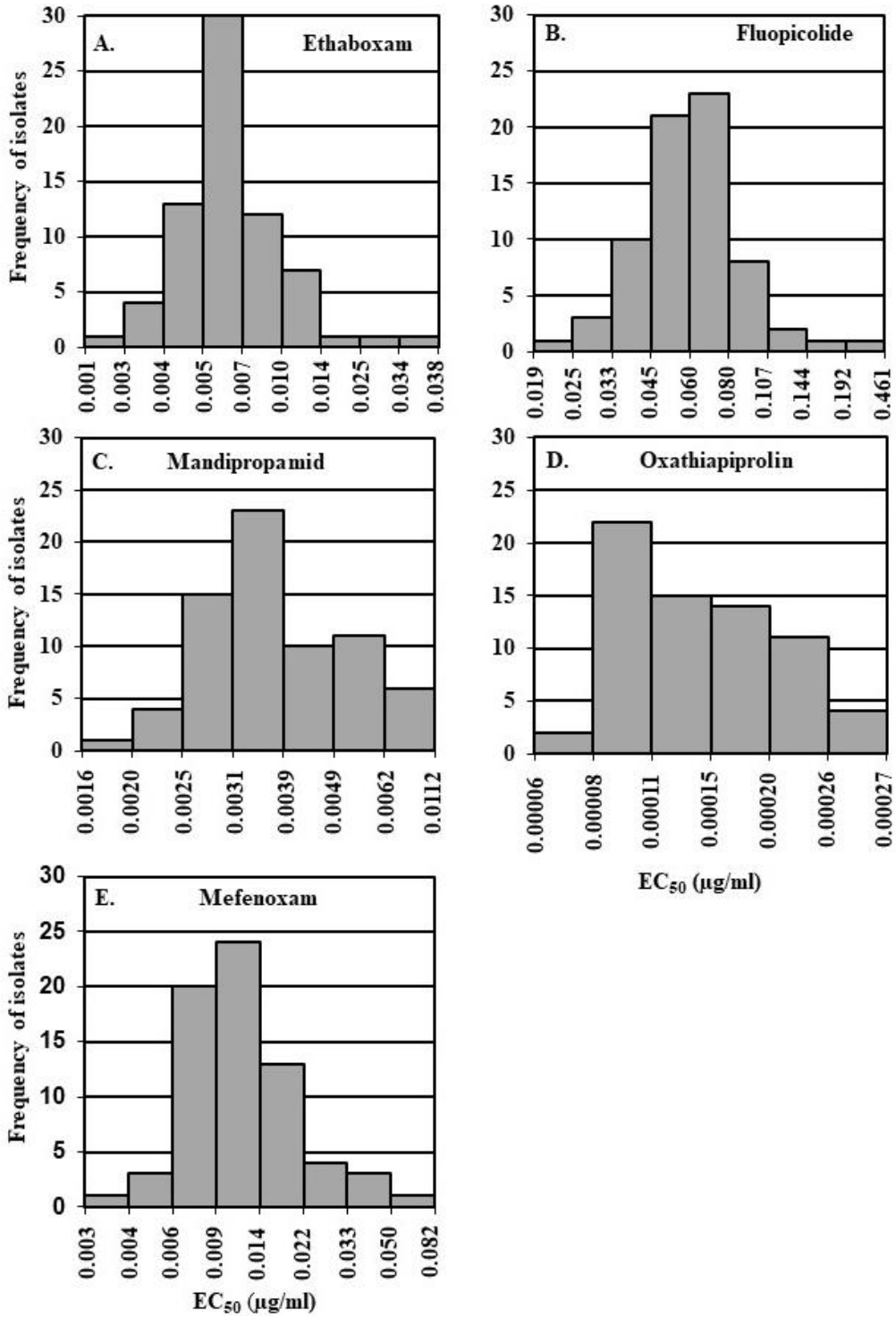

Figure 1.1.2. Frequency histograms of effective concentrations to inhibit mycelial growth of 71 isolates of *Phytophthora syringae* by 50% (EC_{50} values) for **A,** ethaboxam, **B,** fluopicolide, **C,** mandipropamid, **D,** oxathiapiprolin, and **E,** mefenoxam as determined by the spiral gradient dilution method.

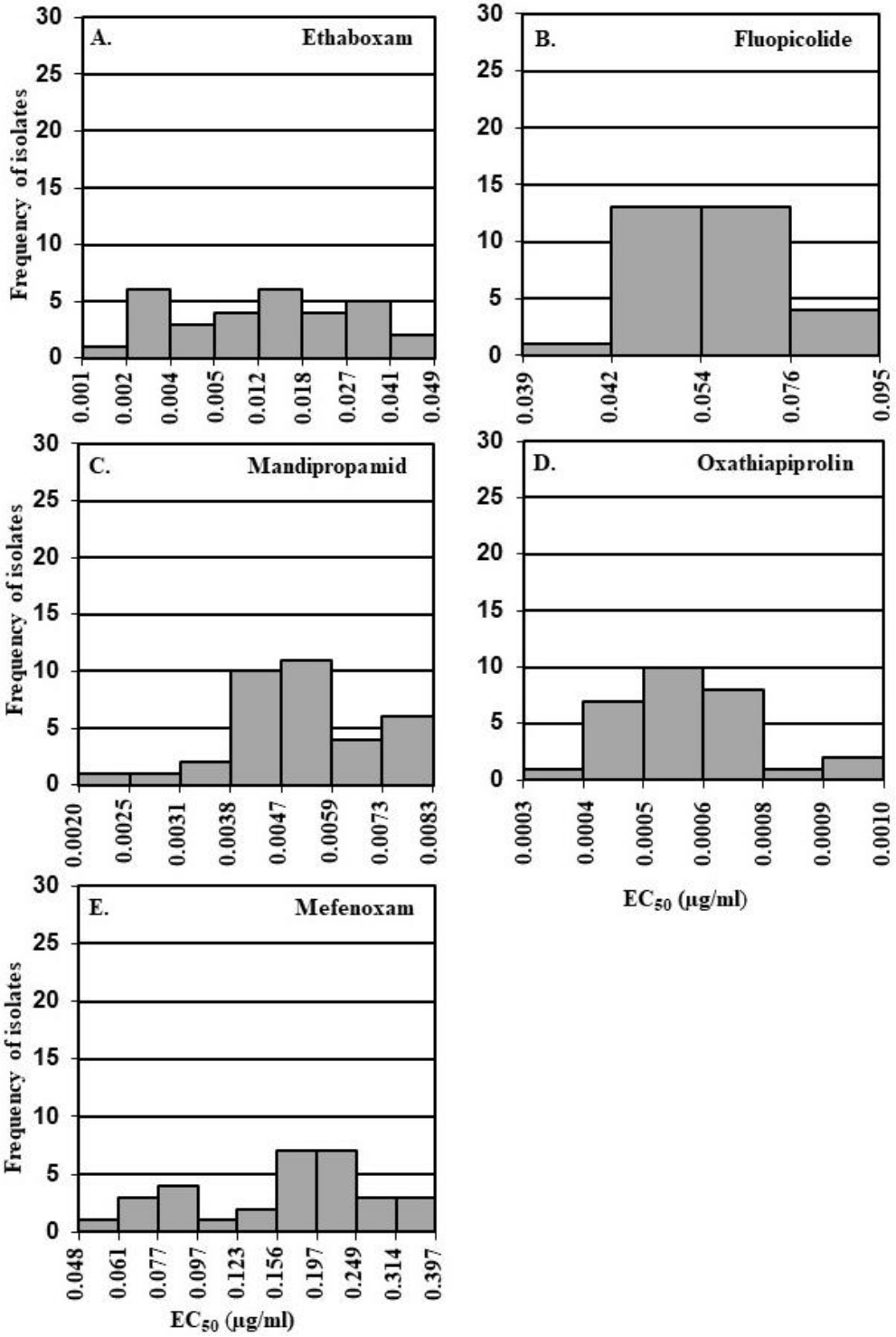

Figure 1.1.3. Frequency histograms of effective concentrations to inhibit mycelial growth of 31 isolates of *Phytophthora nicotianae* by 50% (EC_{50} values) for **A,** ethaboxam, **B,** fluopicolide, **C,** mandipropamid, **D,** oxathiapiprolin, and **E,** mefenoxam as determined by the spiral gradient dilution method.

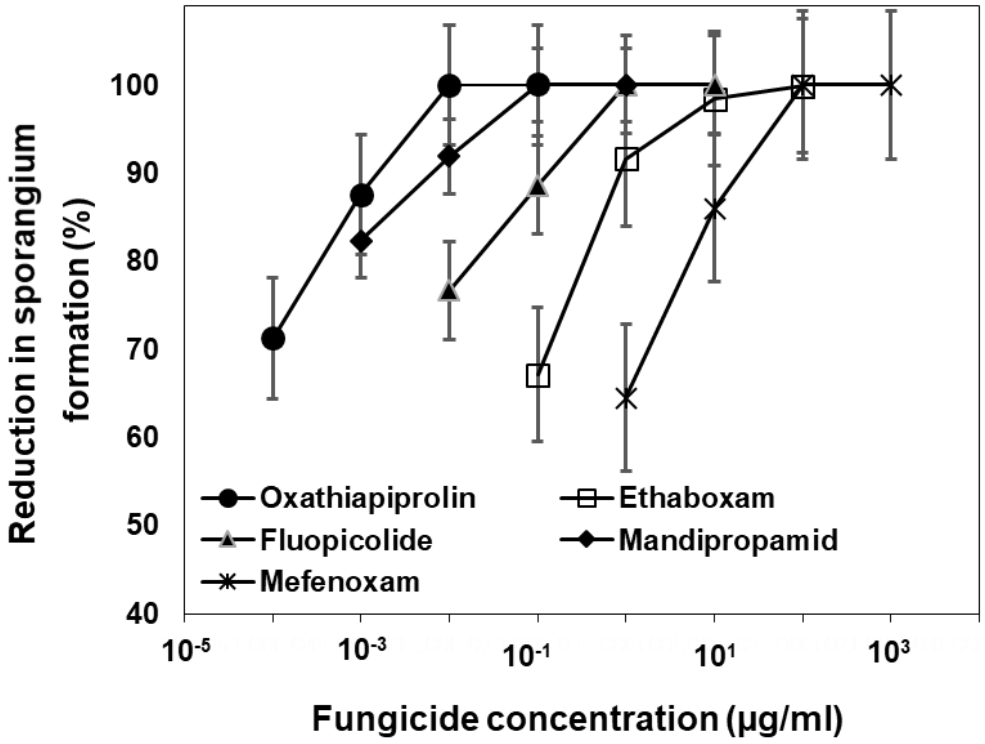

Figure 1.2. Effect of ethaboxam, fluopicolide, mandipropamid, mefenoxam, and oxathiapiprolin on sporangium formation of *P. citrophthora.*

CHAPTER II. 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 five 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.

INTRODUCTION

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 Trifoliate and Trifoliate 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 to 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 et al. 2016; Cortright et al. 2016; Jackson et al. 2010; Ji et al. 2014; Patel 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 2.1) 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 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 2.2 with a total of thirteen 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 one-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 ten 10% V8C agar plugs of *P. citrophthora* cultures (isolates 5475 or 5476, Table 2.1), mixed by shaking, and incubated at 25°C for 4 to 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 fungicide treatment. Fungicides were applied in July 2016 (2 weeks after

inoculation) and in May 2017. Fungicides and rates used are listed in Table 2.2, and fungicides were applied as described above. Four replicate trees were treated with water per block and 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 to 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 to 3 days. *P. nicotianae* colonies were identified by their typical growth pattern (Erwin and Ribeiro 1996), and representative colonies were sub-cultured and verified for species identity using species-specific TaqMan qPCR (Hao et al. 2018). Phytophthora 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 in 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 to 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 m × 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 (The 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 vs. blue) in the software, and the canopy area of each tree (in m^2) was calculated based on the tarp area of 5.96 m^2. 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 to 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 2.1) that was produced as described above. One milliliter suspension (3000 chlamydospores/ml) was added into each of four holes (3-cm-deep, 7 mm in diameter) around each plant resulting in an equivalent of approximately 20 chlamydospores/g soil.

Treatments were applied one week after inoculation. For potassium phosphite, ProPhyt (Helena Chemical Co., Collierville, TN) was used. Greenhouse application rates (Table 2.2) 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 to 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 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 2.1) 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 2.2) were applied as described above after one 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 to 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 and Weisberg 2001) 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 2.3.1). 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. 2.1.A). 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. 2.1.B).

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 2.3.1). Differences in trunk diameters among treated and control trees were observed in July and December 2015 (Table 2.4) 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 2.3.2). 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 2.4). 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: $P = 0.1071$ 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 2.3.2). 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 2.4). In the 2017 evaluation, four 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 2.5). 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. 2.2). 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. 2.2). 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. 2.2).

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. 2.3). 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 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. 2.4). 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 one, 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 vs. 146 g/ha) and oxathiapiprolin (35 g vs. 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 post-infection 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 EC_{50} 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, as well as 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 (Cerkauskas 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 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 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 EC_{50} value for mycelial growth of 0.24 mg/liter that was in the mid-range 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. 2014). 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 EC_{50} values for mycelial growth inhibition of *P. syringae* by fluopicolide and of *P. citrophthora* by ethaboxam were identified that were $\geq$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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Table 2.1. Information on *Phytophthora nicotianae* and *P. citrophthora* isolates used to inoculate citrus plants in field and greenhouse studies

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

[y] Effective concentrations (EC_{50} 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] EC_{50} values for ethaboxam, fluopicolide, mandipropamid, oxathiapiprolin, and mefenoxam were reported in Gray et al. 2018.

Table 2.2. Fungicide treatments evaluated to manage Phytophthora root rot of navel orange under field and greenhouse conditions

	Field studies				Greenhouse studies		
	Rate		*P. nicotianae*	*P. citrophthora*	Rate	*P. nicotianae*	*P. citrophthora*
Treatment	**g / ha**	**g / tree[u]**	**study**	**study**	**(mg / pot)[u]**	**study**	**study**
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	---
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	---
Oxathiapiprolin	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 + oxathiapiprolin	1,122 + 70	3.24 + 0.20	X[z]	---	---	---	---
Mefenoxam + oxathiapiprolin	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 2.3.1 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[z]
Root rot incidence[x]	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
	Error(treatment×time)	132	2948.1				
CFU per gram soil[x,y]	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				
	Treatment×time	22	103441	2.7358	0.0002	0.0144	0.0004
	Error(treatment×time)	132	226861				
Tree trunk diameter[x]	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
	Error(block×time)	636	6009				
	Treatment×time	22	692	3.3289	<0.0001	<0.0001	<0.0001
	Error(treatment×time)	636	6009				

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

[x] 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.

[y] Colony forming units (CFU) were determined by soil plating.

[z] *P* value was adjusted using Greenhouse-Geisser correction.

Table 2.3.2 Univariate repeated-measures analysis of variance (ANOVA)[w] of tree canopy size, fruit collected per tree, and fruit weight per tree 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[z]
Tree canopy[x]	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				
	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				
Fruit number per tree[x]	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		
	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				
Fruit weight per tree[x]	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] 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.

[y] Colony forming units (CFU) were determined by soil plating.

[z] *P* value was adjusted using Greenhouse-Geisser correction.

Table 2.4. 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*

	Tree trunk diameter (mm)[u]			Tree canopy (m^2)[v]		Fruit production			
						Dec 2016		Dec 2017	
Treatment (g / ha)	**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[z]	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)[w]	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)[x]	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)[y]	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)[x]	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 percentage area covered by the tree canopy of a blue standard background using Assess 1.0.

[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] Mefenoxam was applied at 246 g/ha in the first three applications.

[y] Mefenoxam was applied at 123 g/ha in the first three applications.

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

Table 2.5. 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.

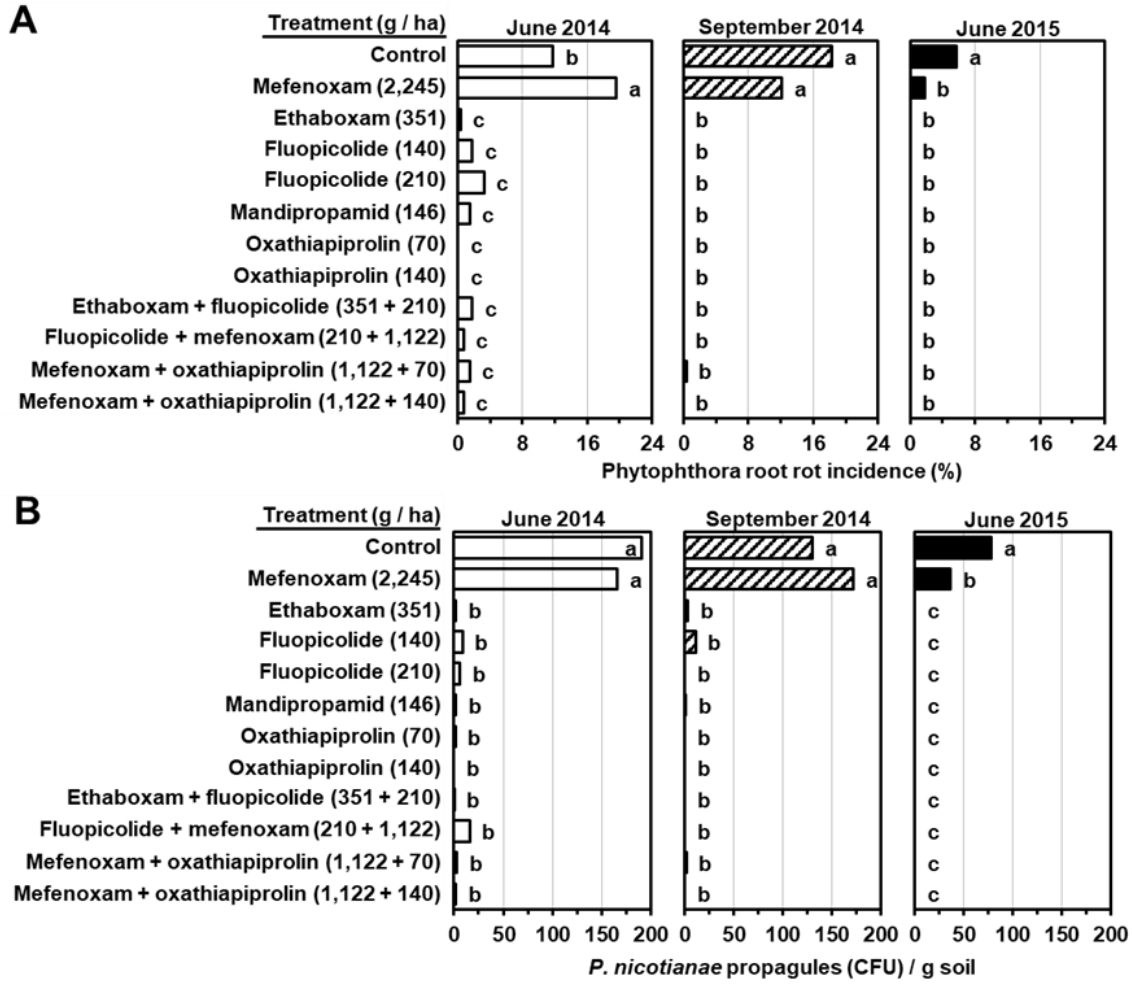

Figure 2.1. Efficacy of fungicides in reducing **A,** incidence of Phytophthora root rot of navel orange on 'Carrizo citrange' rootstock caused by *Phytophthora nicotianae* and **B,** pathogen populations in the soil in a field trial initiated in September 2013. Fungicides were applied in October 2013, 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-oxathiapiprolin (140 g/ha) mixtures, mefenoxam was applied at 246 g/ha in the first three applications, whereas in the mefenoxam-oxathiapiprolin (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 forth application). Bars followed by the same letter do not differ significantly according to Fisher's least significance difference test at $P = 0.05$.

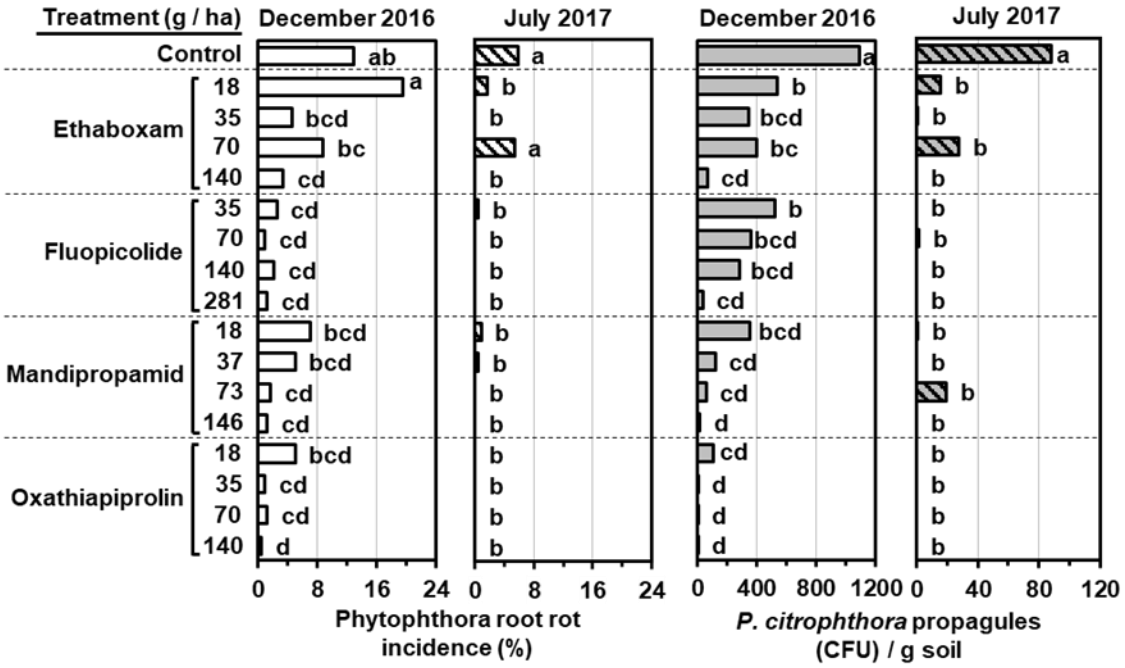

Figure 2.2. Efficacy of selected rates of new Oomycota fungicides in reducing the incidence of Phytophthora root rot of navel orange on 'Carrizo citrange' rootstock caused by *Phytophthora citrophthora* and pathogen populations in the soil in a field trial initiated in June 2016. Fungicides were applied 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 $P = 0.05$.

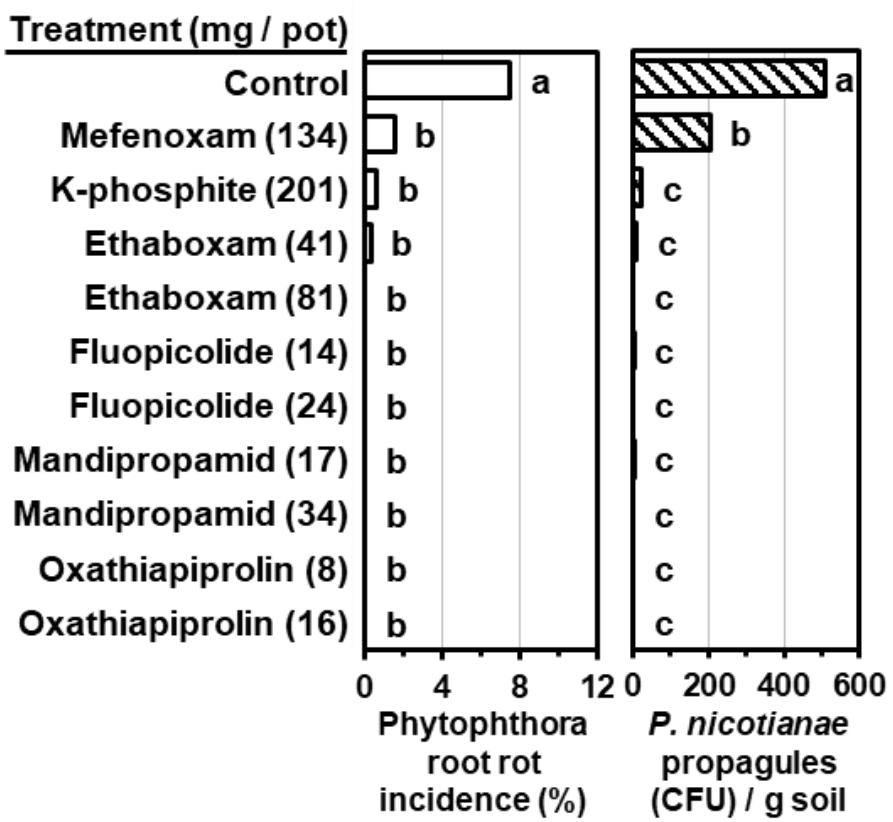

Figure 2.3. Efficacy of new Oomycota fungicides against Phytophthora root rot caused by *Phytophthora nicotianae* in greenhouse studies with 'Madam Vinous' sweet orange seedlings. Fungicides were applied one week after soil inoculation. The incidence of Phytophthora root rot and pathogen soil populations were evaluated after seven 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 $P = 0.05$.

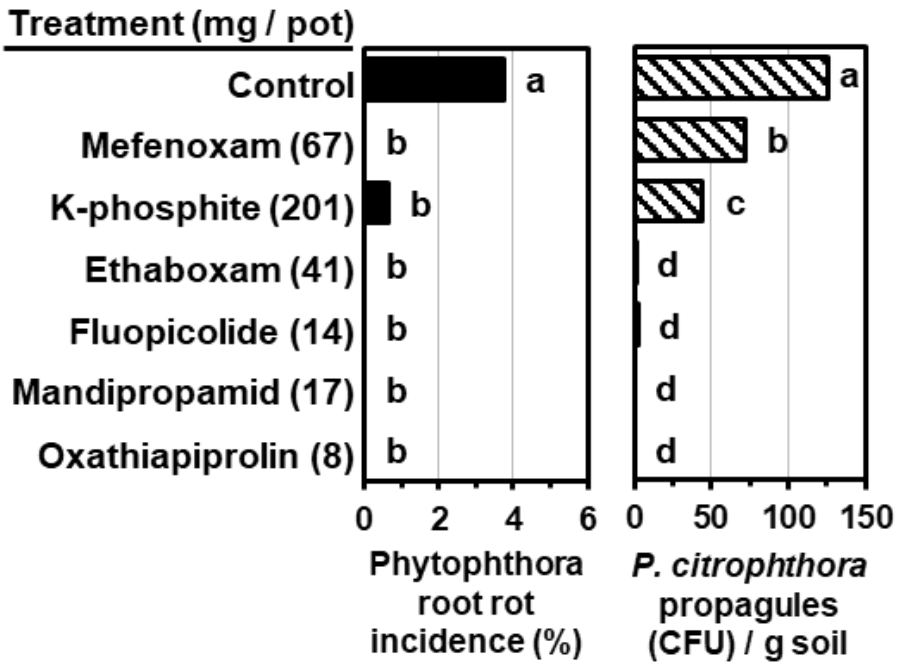

Figure 2.4. Efficacy of new Oomycota fungicides against Phytophthora root rot caused by *Phytophthora citrophthora* in greenhouse studies with 'Madam Vinous' sweet orange seedlings. Fungicides were applied one week after soil inoculation. The incidence of Phytophthora root rot and pathogen soil populations were evaluated after five 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 $P = 0.05$.

CHAPTER III. Mobility of Oxathiapiprolin and Mefenoxam in Citrus Seedlings After Root Application and Implications for Managing Phytophthora Root Rot

ABSTRACT

Oxathiapiprolin is highly effective in the management of Phytophthora root rot of citrus, however, its uptake into plants after soil application is not known. This was investigated and compared to mefenoxam using potted citrus seedlings sampled 7, 10, 13, and 16 days after soil treatments. Bioassays and high performance liquid chromatography-tandem mass spectroscopy (HPLC-MS/MS) were used to quantify fungicide amounts in plant extracts. Distinct inhibition zones of mycelial growth in bioassays with *Phytophthora citrophthora* were observed when root, stem, or leaf extracts were added to filter paper disks on agar plates. Based on bioassays and HPLC-MS/MS quantification of both fungicides, concentrations in the three tissue types and at all sampling times were above the average EC_{50} value of the baseline sensitivity. For oxathiapiprolin, concentrations were significantly higher in roots and leaves than in stems 10 days after treatment, similar after 13 days, and the highest in roots after 16 days. For mefenoxam concentrations were similar in stem and leaf tissues over the experimental period but significantly increased in roots 13 and 16 days after treatment. Regressions of HPLC-MS/MS oxathiapiprolin concentrations on calculated concentrations from bioassay standard curves indicated that the bioassay over-estimated fungicide amounts in the extracts, but the bioassay can be considered a comparable option to costly residue analyses in fungicide mobility studies in plants.

INTRODUCTION

Species of *Phytophthora* cause several diseases on citrus, including root rot, foot rot, brown rot of fruit, and gummosis of tree trunks and larger limbs (Erwin and Ribeiro 1996; Graham and Menge 2000). Phytophthora root rot is common in orchards in California and other citrus production areas worldwide. The disease can be especially damaging in new citrus plantings where overwatering is conducive for infection, and the limited root system of young trees cannot generate new growth fast enough to replace infected and damaged tissues. This can result in poor tree growth and delayed orchard establishment. In California, the disease is mainly caused by *Phytophthora nicotianae* Breda de Haan (syn. *P. parasitica* Dastur) during the warmer months of the year, whereas *P. citrophthora* (R. E. Sm. & E. H. Sm.) Leonian is active year-round (Hao et al. 2018). *P. palmivora* (E. J. Butler) E. J. Butler is the major citrus root rot pathogen in Florida. *P. cactorum* (Lebert & Cohn) J. Schröt., *P. capsici* Leonian, *P. cinnamomi* Rands, *P. drechsleri* Tucker, and *P. megasperma* Drechsler have been occasionally identified in some production areas (Erwin and Ribeiro 1996). Phytophthora root rot is characterized by discoloration and softening of the outer root cortex that becomes water-soaked in appearance and prone to sloughing off, eventually exposing the inner stele. Damage of the root system can lead to tree decline and yield losses from lack of water and nutrient uptake, and if left untreated, to tree death (Graham 1995).

Phytophthora root rot can be managed by cultural practices such as the use of *Phytophthora*-tolerant rootstocks (i.e., ‘Swingle’ citrumelo and Volkamer lemon) (Bright et al. 2004; Ferguson et al. 1990), irrigation or orchard drainage strategies that avoid

overwatering, and fungicide applications. These practices are best used in an integrated approach. Among fungicides, the phenylamides (e.g., metalaxyl, mefenoxam) and the phosphonates (e.g., fosetyl-Al, potassium phosphite) have been used since the 1980s, and until recently, no alternatives were available. The limited number of fungicides registered resulted in their over-use, and in subsequent resistance development. Resistance to the phenylamide class of fungicides has been reported in Oomycota pathogens of numerous crops (Gisi and Sierotzki 2015) including *Phytophthora* spp. that are known to be pathogenic to citrus such as *P. citricola* and *P. nicotianae* (Ferrin and Kabashima 1991; Timmer et al. 1998). Phenylamide-resistant populations of *P. nicotianae* are established in Florida orchards and nurseries (Timmer et al. 1998). Phosphonate resistance is less common but has been identified in isolates of *P. cinnamomi* and *P. infestans* (Wilkinson et al. 2001; Cohen and Samoucha 1984), and more recently in isolates of *P. citrophthora*, *P. syringae*, and *P. nicotianae* from California citrus orchards (Adaskaveg et al. 2017; Förster et al. 2016).

With the need for alternative chemical treatments to manage Phytophthora diseases of citrus, new fungicides have recently become available for evaluation. They include the thiazole carboxamide ethaboxam, the benzamide fluopicolide, the carboxylic acid amide mandipropamid, and the piperidinyl thiazole isoxazoline oxathiapiprolin. Each compound has a unique mode of action that is different from those of the previously registered compounds (Blum et al. 2010; Pasteris et al. 2015; Toquin et al. 2006; Uchida, 2005). Among these, oxathiapiprolin was recently registered on citrus for foliar and soil treatments against Phytophthora diseases. This compound was found to be toxic in vitro

at very low concentrations against several life stages of the pathogens (Gray et al. 2018) and was shown to be highly effective in managing root rot (Hao et al. 2019) and brown rot (Adaskaveg et al. 2017). Belonging to the Fungicide Resistance Action Committee (FRAC) code 49, its mode of action is the inhibition of an oxysterol binding protein, resulting in the inhibition of multiple cellular processes (Pasteris et al. 2016).

Uptake of oxathiapiprolin into citrus plants after soil application is unknown. Previous work has been conducted on annual crops (Cohen 2015; Qu et al. 2016), however, there is currently no information on the mobility and activity of oxathiapiprolin within perennial tree crops. This information may provide a better understanding of its protective and eradicative capabilities in controlling Phytophthora root rot of citrus and have implications on its field use in managing the disease. Therefore, the objectives of this research were to determine if oxathiapiprolin can be detected inside roots and aboveground portions of citrus seedlings (e.g., stems and leaves) after soil application as compared to mefenoxam and if concentrations of the fungicides inside the plants can be effective against *P. citrophthora*. For this, bioassays and analytical residue analyses were performed at selected time periods after treatment of plants.

MATERIALS AND METHODS

Culturing of *P. citrophthora* and fungicides used. *P. citrophthora* (isolate 2440) obtained from citrus in California was cultured on clarified 10% V8 agar (V8C) (Ribeiro 1978) in the dark at 25°C and was stored long-term in liquid nitrogen. Oxathiapiprolin and mefenoxam were used as formulated products (Orondis and Ridomil Gold SL, respectively; both Syngenta Crop Protection; Greensboro, NC) in plant assays and as analytical grades (≥ 98.5 % purity for oxathiapiprolin, ≥ 99.35 % purity for mefenoxam) for standards in the bioassays and analytical residue analyses.

Treatment of citrus seedlings with oxathiapiprolin and mefenoxam. Sweet orange (*Citrus sinensis* (L.) Osbeck) cv. 'Madam Vinous' seedlings in 15 cm x 15 cm x 15 cm pots were grown from seeds in the greenhouse at 24°C to 30°C for 6 to 7 months. At this time, plants were between 25 cm and 30 cm tall. Prior to treatment, plants were moved to an incubator (Model 1-36LLVL; Percival Scientific Inc., Perry, IA) set for a 12-h photoperiod with 34˚C during the light cycle (10,000 to 12,000 Lux) and 26.7˚C during the dark cycle. There were three single-plant replicates for each fungicide treatment and each of the four sample timings (7, 10, 13, and 16 days). Plants were arranged in a randomized complete block design with all four sampling times in each block. Additionally, three replications of untreated control plants were used. Solutions of oxathiapiprolin (1,000 µg/ml) and mefenoxam (2,600 µg/ml) were prepared in distilled water, and 50 ml was added to each pot, resulting in final applications amounts of 50 mg of oxathiapiprolin and 130 mg of mefenoxam per pot. These amounts are comparable to labeled chemigation rate ranges (e.g., 136 g ai/Ha for oxathiapiprolin and 272 g ai/Ha for

mefenoxam) based on the total basin area for 288 trees/Ha and considering that the treatment area of a potted plant is approximately 1/9 of the basin of a newly planted citrus tree. Solutions were added to each pot without wetting the stem, and distilled water was used for the controls. Each pot was then placed in a plastic bag that was tied around the bottom of the stem to reduce evaporation. Plants were watered once nine days after treatment.

Extraction of oxathiapiprolin and mefenoxam from roots, stems, and leaves of citrus seedlings. Plants were harvested 7, 10, 13, or 16 days after treatment. The root ball was shaken to remove most of the soil, washed using tap water, and allowed to air-dry briefly at ambient temperature. Roots were sampled randomly. The stems were cut 1.5 cm above the soil line to avoid fungicide contamination from the soil application. Another cut was done 10 cm above the first cut, and stem and leaf tissues within this stem segment were separated. Tissues were cut finely using a clean razor blade for each plant and tissue. One gram of each tissue was placed into glass scintillation vials (Kimble Glass Inc., Vineland, NJ). The vials were covered with a single layer of cheesecloth and frozen at -80°C for 24 h, lyophilized for 24 h (Labconco Freezone 4.5; Labconco Corp., Kansas City, MO), and then capped and stored at -20°C. A standard procedure was followed for extraction of plant tissues for both fungicides (Anonymous, 2016). For this, the lyophilized tissues were transferred to 2-ml impact resistant tubes (USA Scientific Inc., Ocala, FL) containing two stainless-steel grinding balls (Steelbal Lysing Matrix; MP Biomedicals, LLC, Solon, OH) and pulverized for 60 s using the FastPrep-24 (MP Biomedicals, LLC) set at 6.0 m/s. The contents of each tube were transferred to a 15-ml conical

polypropylene plastic tube (Thermo Fisher Scientific, Waltham, MA) for a single-phase extraction. For this, 1 ml of sterile ultrapure water (ddiH_2O) was added to each tube and the tube was incubated for 5 min to allow soaking of the sample. An additional 800 µl of sterile ddiH_2O, 2.4 ml of acetonitrile, and 20 µl of formic acid (both chemicals: Optima LC/MS grade; Thermo Fisher Scientific) were added, and the tubes were placed on an orbital shaker at 300 rpm for 5 min. The tubes were centrifuged at 1,380 g for 10 min. The supernatant was transferred to a 15-ml plastic tube, stored at -20ºC, and used for determining fungicide activity in a bioassay within 7 days. For analytical residue analyses using HPLC-MS/MS (high performance liquid chromatography-tandem mass spectroscopy), 500 µl of each tissue extract was transferred into a scintillation glass vial, 2 ml of methanol (Optima LC/MS; Thermo Fisher Scientific), and 4.5 ml of 1% formic acid were added, 0.6 ml of the resulting solution was aliquoted into a 2-ml low-absorption vial (Supelco, Bellefonte, PA), and vials were stored at -20°C until analyses. The experiment was done twice.

Bioassays with *P. citrophthora* for determining the activity of oxathiapiprolin and mefenoxam in citrus tissue extracts. To produce zoospores of *P. citrophthora*, cultures were grown for 3 to 7 days at 25°C on 10% V8C agar. Approximately 50 agar plugs (5 mm in diameter) from the edge of each colony were transferred to a 10-cm petri dish, and approximately 20 ml of 10% V8C broth was added to the plates so as not to submerge the plugs completely. Plates were incubated under fluorescent light (2,200 lux; Daylight Full Spectrum, 5,000K, 40W; Osram Sylvania, Danvers, MA) for 24 h at 25°C before the broth was removed. The mycelial mats were rinsed three times with sterile distilled

water. Approximately 20 ml of sterile distilled water was then added to each plate and the plates were incubated under fluorescent light for an additional 24 h to promote sporangial production. Plates were incubated at 4°C for 20 min and at 25°C for another 20 min to induce the release of zoospores. The zoospore suspension was transferred to a 50-ml tube and vortexed for 60 s to cause zoospore encystment. Aliquots of 800 µl of the cyst suspension (2.5×10^4 cysts/ml) were spread onto 10-cm 10% V8C plates and plates were allowed to air-dry in a laminar flow hood for 1 to 2 h. Five 6-mm-diameter glass microfiber filter disks (Whatman GF/A; Sigma Aldrich, St. Louis, MO) were placed equidistantly from each other on the agar surface. Aliquots of 10 µl of extracts were deposited onto each disk, disks were allowed to dry for 5 min, and another 10 µl was added (a total of 20 µl extract per disk). For the control, 20 µl of an acetonitrile-ddiH_2O-formic acid mixture (as described for the tissue extracts above) was deposited onto each disk. Three replicated plates were used for each extract and the control. Diameters of inhibitory zones where no *P. citrophthora* growth was observed were measured after 48 h. The experiment was done twice for each fungicide.

Standard curves were developed using inhibition zones from known amounts of fungicides applied to filter paper disks. For this, 10 µl of aqueous suspensions containing 0.5, 0.25, 0.1, 0.05, or 0.01 µg of mefenoxam or 0.025, 0.01, 0.005, 0.0025, or 0.0005 µg of oxathiapiprolin were deposited onto the disks with three replicated plates for each fungicide concentration. Diameters of inhibitory zones were measured as described above and the experiment was done three times.

Detection of oxathiapiprolin and mefenoxam by HPLC-MS/MS. Analytical grade oxathiapiprolin and mefenoxam (Chemservice, West Chester, PA) were dissolved in acetonitrile and serially diluted. The samples were analyzed for oxathiapiprolin and mefenoxam using a standard curve method. The concentration of standards (prepared in 1% formic acid: methanol 70:30, v/v) used for quantitation were 0.1, 0.5, 1.0, and 10 ng/ml. Each dilution was transferred to a 2-ml low-absorption vial, and aliquots were transferred to auto-sampler vials for analysis that was performed by Environmental Micro Analysis Inc. (Woodland, CA). The autosampler vials were analyzed using high-pressure liquid chromatography (Model 1290; Agilent, Santa Clara, CA) coupled with tandem electrospray mass spectrometer (Model API 4000AB; SCIEX, Concord, Canada). The chromatographic separation was achieved on a XB-C18 HPLC column (50 x 4.6 mm, 100Å, 2.6 µm; Phenomenex; Torrance, CA). The samples were analyzed with standard concentration levels indicated above. The limit of detection was calculated using decreasing concentrations for mefenoxam and oxathiapiprolin as 0.1 ng/ml. The mass transitions used for oxathiapiprolin were 540→500, 540→163.1, and 539→499. The mass transitions used for mefenoxam were 280→220, 280→192, and 280→248.

Oxathiapiprolin concentrations were determined for the three tissue types for all timings, whereas for mefenoxam, concentrations were only quantified for the 13-day sampling. For each tissue type and sampling time, a composite extract sample from three plants was submitted from each of two repeated experiments.

Statistical analysis of data. For bioassay standard curve data, linear regression analyses (PROC REG; SAS ver. 9.4; SAS Institute, Cary, NC) of inhibition zones of mycelial

growth of *P. citrophthora* on known amounts of oxathiapiprolin or mefenoxam applied to filter disks were performed following $\log_{10}$ transformations of inhibition zones (mm) and active ingredient of fungicide/disk (µg).

For bioassays using plant extracts with unknown amounts of fungicides, the dependent variable was the $\log_{10}$-transformed mean inhibition zone; whereas for plant extracts analyzed using HPLC-MS/MS, the dependent variable was the $\log_{10}$-transformed mean amount of fungicide calculated per g of tissue. These data were analyzed using generalized linear mixed models with the GLIMMIX procedure of SAS. For this, root, stem, and leaf extracts or days after treatment were treated as fixed effects, and experiment, replication (block), and the overall error term were treated as random effects. Fixed effects were tested for significance by days after treatment or by tissue being analyzed, and least squares treatment means were constructed using the lsmeans statement with the Tukey adjustment in PROC GLIMMIX. Contrast estimates for pairs of treatments were used to determine multiple comparison differences among treatment means.

For comparisons of the HPLC-MS/MS and bioassay data for concentrations of oxathiapiprolin in plant extracts, regression analyses were performed using PROC REG of SAS. For this, concentrations (µg/g tissue) of oxathiapiprolin in plant extracts determined by HPLC-MS/MS were regressed on those calculated from bioassays using the standard regression curve equation in Fig. 3.2A. Equations of the standard curves were used to first calculate the amount of fungicide in 20 µl plant extract that was applied to the filter disk. These values were then adjusted with a dilution factor of 211 for

bioassay data or a factor of 59 for HPLC-MS/MS data (ng/ml) to account for the total amount of fungicide obtained from 1 g of tissue.

RESULTS

Bioassays with *P. citrophthora* for determining the activity of oxathiapiprolin and mefenoxam. Growth inhibition of *P. citrophthora* zoospore cysts was observed on bioassay plates around filter paper disks treated with oxathiapiprolin or mefenoxam, and the width of the inhibition zone after two days of incubation depended on the amount of fungicide applied (Fig. 3.1). No inhibition zones were observed in the controls and the lowest amounts of each fungicide tested. In regression analyses, there was a linear relationship between $\log_{10}$-transformed mean inhibition zones and amounts of fungicide applied to the disks with R^2 values of 0.9197 for oxathiapiprolin (Fig. 3.2A) and 0.9359 for mefenoxam (Fig 3.2B). Equations were calculated for both regression lines (Fig. 3.2A,B).

Quantification of oxathiapiprolin in citrus tissue extracts using bioassays and HPLC-MS/MS. Distinct inhibition zones in bioassays with *P. citrophthora* were also observed when root, stem, or leaf extracts of potted plants that were treated using soil applications were added to the filter paper disks. Inhibition zone sizes of extracts from oxathiapiprolin-treated plants depended widely on tissue type and sampling time after treatment (Fig. 3.3A). Mean inhibition zones of extracts prepared 7 days after treatment were less than 3 mm wide, and there was no significant ($P = 0.1923$) difference for the three tissue types. Significant differences among tissues, however, were observed at 10

($P < 0.0001$) and 16 ($P = 0.033$) days, but not 13 days, after treatment. Thus, at 10 days, inhibition zones of root and leaf extracts were wider than for stem extracts, and at 16 days, inhibition zones for root extracts were the largest (Fig. 3.3A). In comparison of sampling times, inhibition zones for root extracts were not significantly different. Zones for stem extracts were significantly ($P = 0.037$) wider for extracts prepared 13 days after treatment than for the 7- or 10-day extracts. Leaf extracts were significantly ($P = 0.003$) the most inhibitory when prepared after 13 days, but least inhibitory after 7 and 16 days.

Quantification of oxathiapiprolin residues by HPLC-MS/MS overall followed a similar trend among sampling times and tissue types (Fig. 3.3B) as the bioassays but 7 days after treatment, concentrations in root extracts were significantly ($P = 0.044$) higher than those in stems or leaves. Similar to the bioassay, significant differences among tissues were observed at 10 ($P < 0.001$) and 16 ($P = 0.021$) days, but not 13 days, after treatment. Again, at 10 days, concentrations in root and leaf extracts were higher than in stem extracts, and at 16 days, those in root extracts were the highest (Fig. 3.3B). Over the sampling period, concentrations in root extracts were significantly ($P = 0.0074$) the highest 16 days (i.e., 1.56 µg/g) and lowest 7 days (i.e., 0.51 µg/g) after treatment. There was no statistical difference ($P= 0.1444$) in stem extract concentrations over the sampling period (concentrations range from 0.06 µg/g to 0.37 µg/g), and concentrations in leaf extracts were significantly ($P = 0.0224$) higher 10 (i.e., 0.86 µg/g) and 13 days (i.e., 1.15 µg/g) after treatment as compared with 7 (i.e., 0.07 µg/g) or 16 (i.e., 0.06 µg/g) days after treatment. No oxathiapiprolin was detected in any of the untreated plant samples.

Oxathiapiprolin amounts in extracts that were applied to filter disks in the bioassays were calculated based on the inhibition zones in Fig. 3.3A using the regression equation obtained in Fig. 3.2B. When these values were regressed on oxathiapiprolin concentrations obtained for the same extracts using HPLC-MS/MS, linear relationships were obtained with R^2 values of 0.9534 for root, 0.9678 for stem, and 0.783 for leaf extracts (Fig. 3.4). Slopes for the regression lines were 0.358 for leaf, 0.4385 for stem, and 0.7151 for root extracts.

Quantification of mefenoxam in citrus tissue extracts using bioassays and HPLC-MS/MS. In the temporal comparison of the inhibitory action of root, stem, and leaf extracts in bioassays with *P. citrophthora*, there was no significant ($P \geq 0.208$) difference in inhibition zones around filter disks for the three types of extracts obtained 7 and 10 days after soil applications with mefenoxam to citrus seedlings (Fig. 3.5). Root extracts prepared 13 or 16 days after application, however, resulted in significantly ($P < 0.0001$) wider inhibition zones than extracts from stems and leaves. Unlike in the bioassay with root extracts from oxathiapiprolin-treated plants, inhibition zones from mefenoxam-treated plants increased significantly from 13 days to 16 days ($P < 0.0001$) after treatment when the overall largest mean inhibition zone of 17.6 mm was obtained.

HPLC-MS/MS analysis of extracts of mefenoxam-treated plants was only done for the 13-day sampling. Mean concentrations for root, stem, and leaf tissues were 649.1 µg/g, 37.7 µg/g, and 100.7 µg/g, respectively, and there were significant ($P < 0.0001$) differences between all three tissue types.

DISCUSSION

In this study, we investigated the comparative mobility of oxathiapiprolin and mefenoxam in citrus seedlings after soil applications with the fungicides. Our data indicate that the newly registered oxathiapiprolin can be taken up by citrus roots and be translocated into aboveground plant parts. With this, oxathiapiprolin has similar properties as the previously registered mefenoxam that we confirmed to be taken up and translocated in plants. Uptake of oxathiapiprolin and mefenoxam in seedling roots increased from 7 to 16 days after application. For oxathiapiprolin, concentrations also increased in leaves from 7 to 13 days after treatment, and the reduction at the 16-day sampling point cannot be easily explained. Although much higher absolute concentrations of mefenoxam were detected by HPLC-MS/MS in plant tissues as compared with oxathiapiprolin, inhibition zones in the bioassays were in a similar range for both fungicides. This can be explained by the much higher in vitro toxicity of oxathiapiprolin for inhibiting mycelial growth as compared with mefenoxam. Thus, in previous research we determined average EC_{50} values for 62 isolates of *P. citrophthora* as 0.0003 µg/ml for oxathiapiprolin and 0.039 µg/ml for mefenoxam (Gray et al. 2018). Concentrations of oxathiapiprolin in the three tissue types were all above the average EC_{50} value of the baseline sensitivity, and thus, are inhibitory to growth of *P. citrophthora*.

Earlier research by others indicated that oxathiapiprolin can be readily absorbed through the epicuticular wax layer of plant leaves, and once inside, translaminar and acropetal movement occurs that provides protection to infection of existing and new leaf

growth (Pasteris et al. 2015). Several studies on the systemic uptake of oxathiapiprolin were conducted with annual vegetable crops. For example, when oxathiapiprolin was applied to the upper surface of detached cucumber leaves, a subsequent reduction of downy mildew on the lower side of the leaf was observed (Cohen 2015). Furthermore in the latter study, application of oxathiapiprolin to the soil of potted cucumber plants provided complete protection against downy mildew infection of existing leaves. In experiments with bell pepper, others demonstrated that following hydroponic or soil applications, oxathiapiprolin was translocated into stems and leaves (Qu et al. 2016). Our study is the first to demonstrate mobility of the fungicide in a perennial plant, and the results have practical implications for the use of oxathiapiprolin. Uptake of the compound as determined by its presence in roots and subsequent acropetal movement into aboveground plant parts indicates that the fungicide has the potential to have long residual activity and persistence in tree crops. This in fact has already been demonstrated in studies on Phytophthora root rot of citrus (Hao et al. 2019) and avocado (Belisle et al. 2019) where bi-annual applications in the field or single applications in the greenhouse provided long-term and highly effective disease control. The mobility of oxathiapiprolin within the root system including basipetal movement will have to be investigated in future studies, possibly using plants with split root systems.

The systemic mobility of metalaxyl and its R-stereoisomer-enriched formulation mefenoxam was previously described in several studies using a variety of plants including tomato and avocado (Zaki et al. 1981), potato (Bruin et al. 1982), and rapeseed (Stone et al. 1987). Metalaxyl treatments to soil of citrus seedlings prior to stem

inoculations with *P. nicotianae* and *P. citrophthora* reduced lesion sizes by 79% and 88% respectively, suggesting movement into the stems (Farih et al. 1981). In another study, drench applications with metalaxyl to young grapefruit trees resulted in the inhibition of *P. nicotianae* when zoospores were exposed to trunk and leaf tissues, further suggesting systemic uptake and activity (Timmer 1979).

Evidence for translocation of oxathiapiprolin in previous studies was based on disease protection of untreated parts of the plant along with the usage of HPLC (Qu et al. 2016) and HPLC coupled with mass spectrometry (Qu et al. 2016; Yu et al. 2017) to confirm residues. Using these studies as precedent, we also utilized HPLC-MS/MS along with bioassays to quantify oxathiapiprolin in plant extracts, while mostly using bioassays for the screening of mefenoxam uptake over time. The HPLC-MS/MS procedures used for this study are current standards accepted by the United States Environmental Protection Agency for measuring residues of oxathiapiprolin and mefenoxam. The bioassay and analytical methods resulted in very similar relative differences among tissues and sample times. Absolute concentrations that were calculated from bioassay inhibition zones using a standard curve, however, were over-estimated with regression slopes between 0.358 and 0.715 as compared to detection by HPLC-MS/MS. Ideally, a regression slope of 1 would be expected for both methods to be equivalent. Still, the bioassay used can be considered a comparable option to costly residue analyses in fungicide mobility studies in plants.

In summary, our results demonstrate the potential of oxathiapiprolin to be translocated within citrus plants. Studies were conducted with seedlings that were

incubated at relatively high temperatures, and pots were placed inside plastic bags to reduce evaporation and minimize the need for frequent watering. Still, a proportional amount of fungicide was applied within the labeled rates of both fungicides for citrus. In mature trees, detection of the fungicide in the canopy is unlikely due to the size of the tree, dilution of the fungicide, and low labeled rates for soil applications. This is inconsequential, however, to root and foot rot management as long as the fungicide is taken up by roots, distributed in the root system, moves into the crown, and persists for some time to protect the tree from *Phytophthora* infections that are most damaging to tree health.

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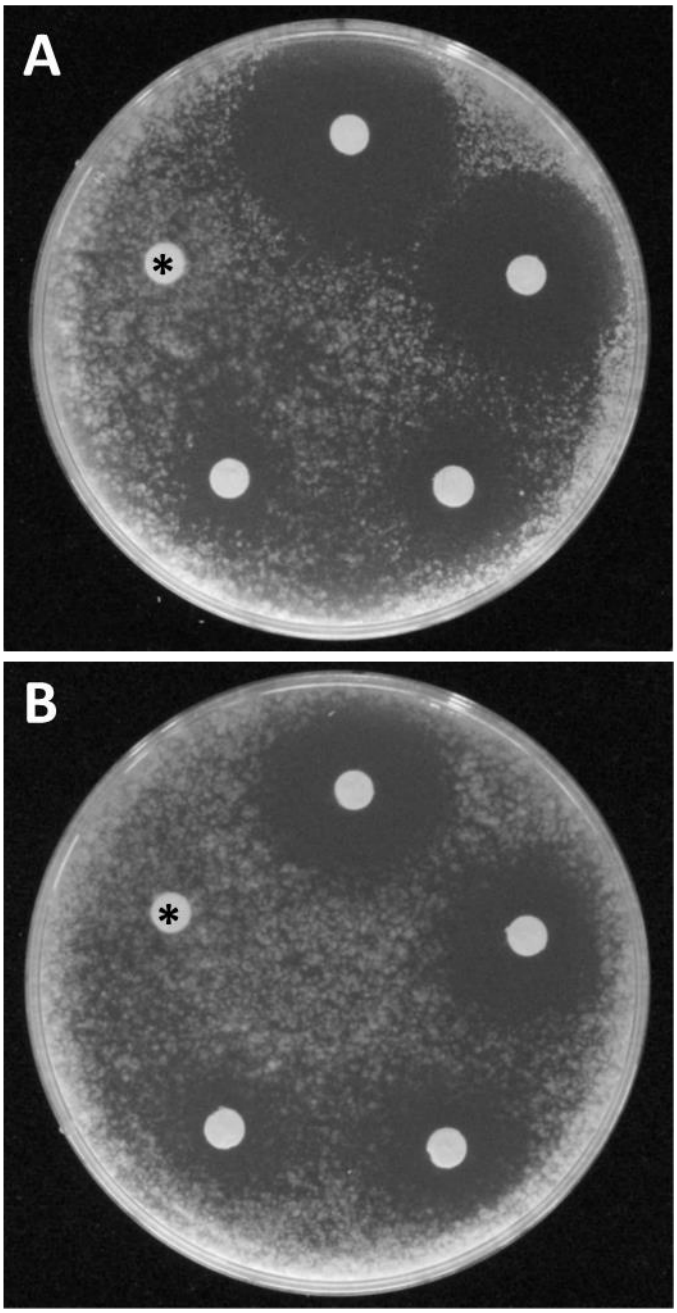

Fig. 3.1. Inhibition zones of mycelial growth of *Phytophthora citrophthora* in a bioassay with standard concentrations (10 µl/6-mm-diameter disk; counter-clockwise from the asterisks) of **A**, oxathiapiprolin (0.0025, 0.005, 0.01, 0.025, 0.05 µg/ml) and **B**, mefenoxam (0.01, 0.05, 0.1, 0.25, 0.5 µg/ml) after two days of incubation at 24°C.

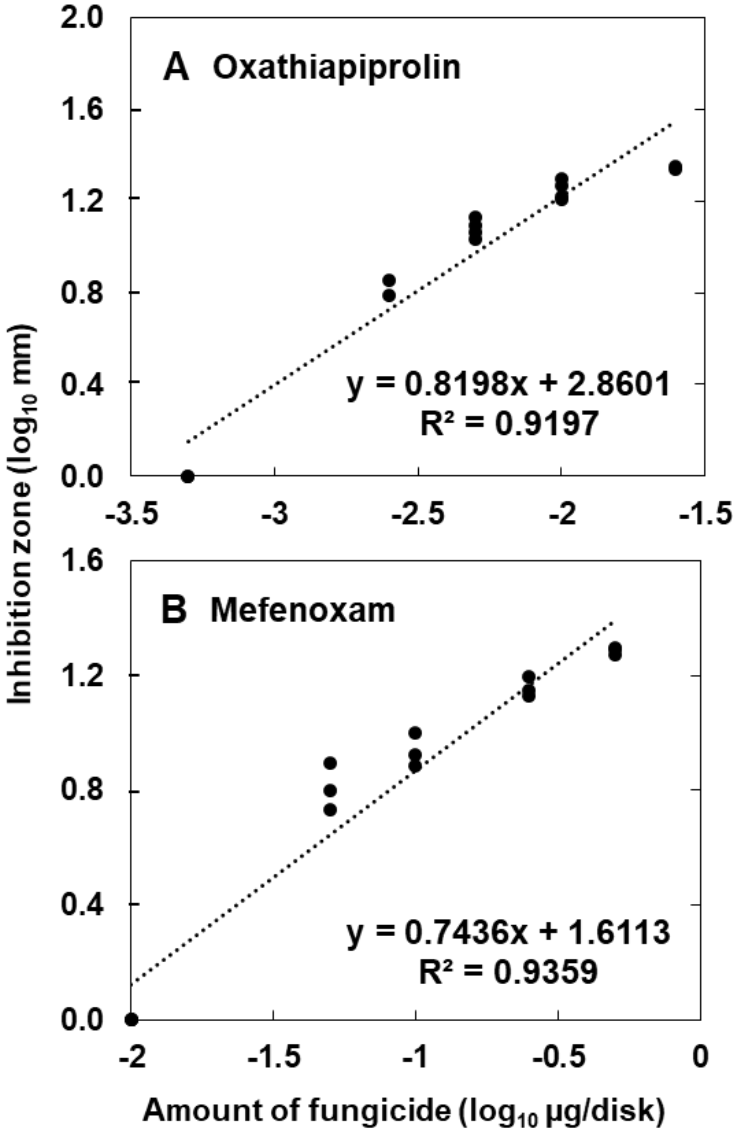

Fig. 3.2. Regressions of mean inhibition zones of mycelial growth of *Phytophthora citrophthora* on known amounts of **A**, oxathiapiprolin or **B**, mefenoxam using a bioassay.

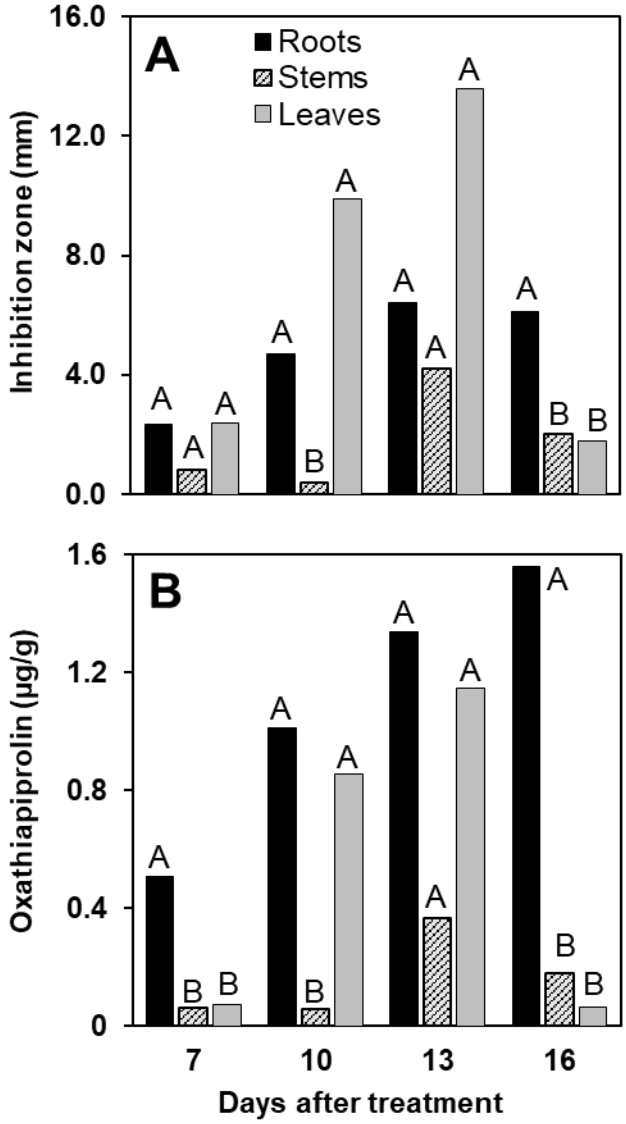

Fig. 3.3. A, Mean inhibition zones of mycelial growth of *Phytophthora citrophthora* in a bioassay and **B**, oxathiapiprolin concentrations measured in the same extracts by HPLC-MS/MS analysis in root, stem, and leaf extracts of citrus plants treated with soil applications of oxathiapiprolin and sampled after 7, 10, 13, or 16 days. Bars followed by the same letter within each sampling time are not significantly different according to GLIMMIX analysis and least square treatment means with the Tukey adjustment.

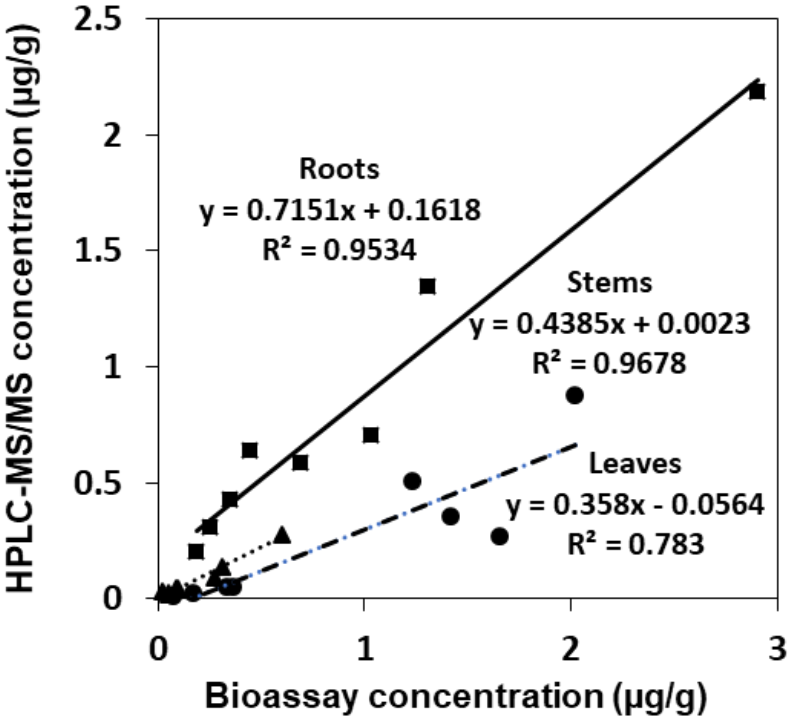

Fig. 3.4. Regressions of concentrations of oxathiapiprolin in plant extracts determined by HPLC-MS/MS on those calculated from bioassays. For the bioassays, fungicide amounts in plant extracts were calculated using the standard curve equation in Fig. 2A. ■ = Roots, ● = Leaves, and ▲ = Stems.

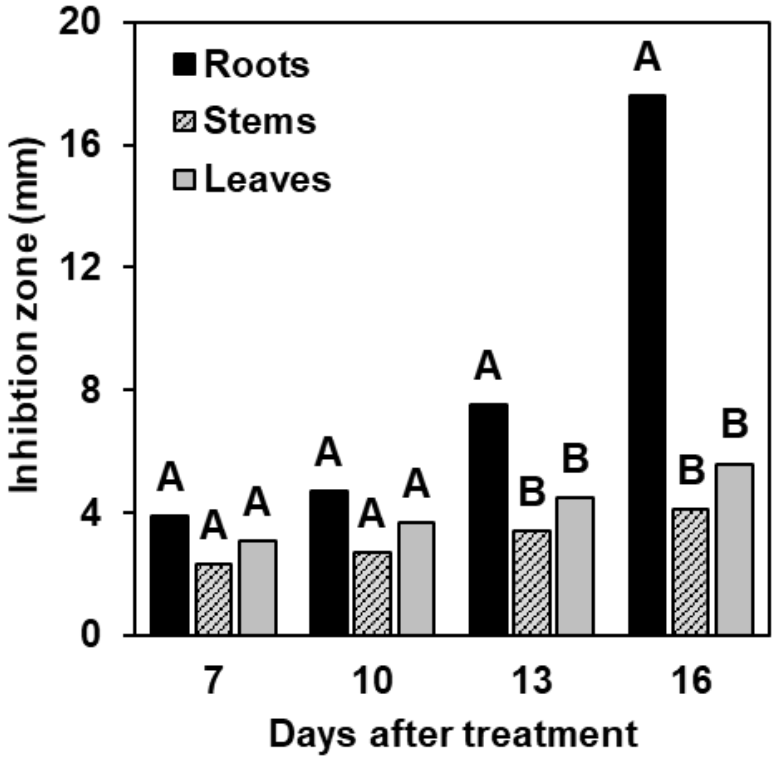

Fig. 3.5. Mean inhibition zones of mycelial growth of Phytophthora citrophthora in a bioassay in root, stem, and leaf extracts of citrus plants treated with soil applications of mefenoxam and sampled after 7, 10, 13, or 16 days. Bars followed by the same letter within each sampling time are not significantly different according to GLIMMIX analysis and least square treatment means with the Tukey adjustment.

GENERAL CONCLUSION

The successful control of Phytophthora diseases in citrus production relies on both cultural and chemical methods to form a well-developed integrated management system. The latter of these has for many years, seen a lack of new compounds introduced, leading to resistance development in long used treatments and making these less effective as a result. The studies included within this dissertation verify the efficacy of several new fungicides for the control of Phytophthora diseases of citrus within California through the evaluation of ethaboxam, fluopicolide, mandipropamid, and oxathiapiprolin. This work is the first to compare the invitro toxicity of these fungicides to several different life stages of multiple *Phytophthora* spp. recovered from important citrus growing regions within California using *Phytophthora nicotianae, P. citrophthora*, *P. syringae* and *P. hibernalis* and further, to determine baseline sensitivity ranges of these isolates. This work is also the first to investigate the suitability of these fungicides as chemical management solutions for the control of Phytophthora root rot of citrus as well as their potential efficacy following alterative rate applications, of which several were found to be successful in reducing both Phytophthora root rot incidence and soil populations at both full and reduced rates. Finally, the systemic properties of oxathiapiprolin within citrus seedlings was investigated to better determine the full range of the Oomycota fungicide's protective capabilities and any potential application practices that could be observed to improve the management of additional Phytophthora diseases, such as gummosis.

The lack of a wide range of available fungicides for the control of Phytophthora diseases of citrus has been a long standing issue that until recently, had seen no new chemistries proposed for field usage. Of the fungicides studied to determine their efficacy at controlling several different life stages of *Phytophthora* species pathogenic to citrus, oxathiapiprolin was found to be the most efficacious and highly effective at extremely low concentrations ($\leq$0.010 µg/ml) to all life stages screened. Oxathiapiprolin, along with mandipropamid, were the only two fungicides found to be capable of inhibiting all life stages examined, including mycelium, sporangial formation, zoospore cyst germination, oospore and chlamydospore formation. The other fungicides ethaboxam and fluopicolide were similarly effective at controlling mycelial growth and exhibited low inhibitory values for both mycelial growth and sporangial production of *Phytophthora* species from citrus, similar to previous studies of several *Phytophthora* spp. by others. Zoospore cyst germination was however, found to be insensitive to ethaboxam and fluopicolide. In comparison, the mycelial growth of all 166 isolates screened was found to be sensitive to the four new fungicides, with similar EC_{50} values reported for the four species studied across each fungicide, providing a valuable record for future disease monitoring efforts. This establishes the potential of these fungicides to provide effective management of important life stages necessary to the spread of Phytophthora diseases of citrus and other crops.

Greenhouse and field trials to determine the efficacy of the four new fungicides were conducted to further verify the potential of these fungicides for the management of Phytophthora root rot. From these studies ethaboxam, fluopicolide, mandipropamid and

oxathiapiprolin were identified as highly effective at not only reducing Phytophthora root rot incidence but also soil Phytophthora populations in both greenhouse and field studies, in comparison to the fungicides mefenoxam and potassium phosphite which are industry standards for Phytophthora disease management. Positive trends were observed in increased trunk diameters, canopy volume and crop yield in treated plants versus controls, with fluopicolide and oxathiapiprolin identified as the most successful fungicides at promoting positive growth trends, likely due to reduced disease pressure in treated trees. In a field study conducted with *P. citrophthora* aimed at determining potential lower application rates, alternative rates of mandipropamid and oxathiapiprolin (73 and 35 g/ha, respectively) were determined to be just as effective at inhibiting Phytophthora root rot and soil populations in comparison to earlier field trials conducted with *P. nicotianae* in which application rates matched those suggested by their respective registrants at 146 g/ha for mandipropamid and 70 g/ha for oxathiapiprolin. The high efficacy of these fungicides in reducing disease at lowered rates correlated with their low effective concentrations (EC) values that had been determined previously, verifying their suitability for field usage as a means of Phytophthora root rot management.

Oxathiapiprolin, which in these studies was found to be the most effective fungicide at inhibiting the growth of several *Phytophthora* species invitro, and in greenhouse and field trials, was analyzed and compared against mefenoxam following application of the differing fungicides to root systems of citrus seedlings using bioassays and HPLC-MS/MS (high performance liquid chromatography-tandem mass spectroscopy) to better understand it's protective capabilities. It was unknown whether

oxathiapiprolin could move systemically within citrus plants to the upper portions of the plant following treatment, though previous studies in other plant systems suggested this to be likely. In plants treated with oxathiapiprolin, detection of the fungicide was examined over 7, 10, 13 and 16 days in root, stem and leaf tissue following both bioassays and HPLC-MS/MS analysis conducted with the same plant extracts. Extracts from oxathiapiprolin treated plants yielded measurable zones of inhibition in the mycelial growth of *Phytophthora citrophthora* from root, stem and leaf tissue following bioassay screens. In support of these results, HPLC-MS/MS tests with the same extracts of roots, stems and leaves of treated plants first screened with bioassays resulted in the recovery of oxathiapiprolin from all tissue types and at all times, in concentrations above the EC_{50} value previously determined in baseline sensitivity screens. The estimated content of oxathiapiprolin differed by tissue type and day, with concentrations found to be significantly higher in the root and leaf tissue at 10 days after treatment and less in the stem. This was similar at 13 days and at 16 days, root retention of oxathiapiprolin was the highest of all the plant tissues tested for that period. Mefenoxam concentrations were found to be the most similar in stem and leaf tissues over all the time points tested, with concentrations in the roots increasing at 13 and 16 days after treatment. Although calculated concentrations from the bioassays conducted for oxathiapiprolin were regressed against HPLC-MS/MS results of the same various tissue types and suggested the bioassay tended to overestimate fungicide amounts, this methodology is still a viable and more cost effective method in comparison to analytical residue analysis to determine fungicide uptake and mobility. These results indicate the application of oxathiapiprolin to

the root systems of citrus plants readily encourages uptake. They, along with the HPLC-MS/MS quantification, also show that oxathiapiprolin is subsequently retained within the root systems, confirming that it's retention in these tissues make it highly effective as a Phytophthora root rot treatment.