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An Integrated Study of the Avocado Root Rot Pathogen *Phytophthora cinnamomi*

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by

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DEDICATION

I would like to dedicate this dissertation to all of my friends and family who helped me throughout my PhD. To my parents who have always been there for me. To my son Jack for inspiring me to work harder so I can provide for him and set a good example. Finally, I would like to dedicate this dissertation to my beautiful wife, Wei, who has always believed in me, supported me, and helped me to be a better person.

ABSTRACT OF THE DISSERTATION

An Integrated Study of the Avocado Root Rot Pathogen
Phytophthora cinnamomi

by

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Doctor of Philosophy, Graduate Program in Plant Pathology
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Dr. Patricia Manosalva, Chairperson

Phytophthora root rot (PRR), caused by *Phytophthora cinnamomi* (*Pc*), is the most destructive disease of avocado worldwide. This study described the phenotype of a subset of *Pc* isolates representing two, previously identified, genetically distinct clades of A2 mating type isolates in California regarding growth rate, optimal growth temperature, virulence, and fungicide sensitivity. Isolates corresponding to the clade I group exhibited higher mycelial growth rate and were more sensitive to higher temperatures than clade II. Isolates that were more virulent in avocado and less sensitive to potassium phosphite were also identified. A detached leaf *Pc* inoculation method using *Nicotiana benthamiana* was developed and validated providing an alternative method for assessing the virulence of a large number of isolates.

Seventy-one isolates of *Pc* collected from avocado growing regions in California were evaluated for their *in vitro* sensitivities to four new Oomycota fungicides ethaboxam, fluopicolide, mandipropamid, and oxathiapiprolin and two registered fungicides mefenoxam and potassium phosphite. The four new fungicides and

mefenoxam showed high *in vitro* toxicities with relatively low mean effective concentrations to inhibit mycelial growth by 50% (EC_{50}) values. Isolates with reduced sensitivity to potassium phosphite were identified. Greenhouse trials were conducted to assess the efficacy of these fungicides for managing PRR on avocado seedlings and rootstocks. Mefenoxam and potassium phosphite were effective treatments, however, oxathiapiprolin, fluopicolide, and mandipropamid were more effective.

RNA sequencing (RNAseq) transcriptome analysis of *Pc* infected *N. benthamiana* leaves at 6, 12, 24, 36, and 48 hours post-inoculation was performed. Genes involved in hormone signaling, receptor-like kinases, NBS-LRRs, transcription factors, secondary metabolism, and production of antimicrobial compounds were differentially expressed (DE). Major up-regulated biochemical pathways included the biosynthesis of secondary metabolites and plant-pathogen interactions. Major down-regulated biochemical pathways were primary metabolic pathways and carbon metabolism. Among the DE genes identified, a jasmonic acid responsive gene cytochrome P450 was up-regulated 13.3-fold at 36 hpi, and a gene encoding for a salicylic acid binding protein was down-regulated 10.5-fold. WRKY transcription factor 51, up-regulated by 13.5-fold at 24 hpi, was transiently overexpressed in *N. benthamiana* and significantly decreased the lesion development on *Pc* inoculated leaves compared to the GFP control.

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CHAPTER 1. GENERAL INTRODUCTION

The oomycete *Phytophthora cinnamomi* Rands is the causal agent of Phytophthora root rot (PRR) of avocado, the most destructive disease in the avocado industry worldwide (Erwin and Ribeiro 1996). PRR affects approximately 75% of California avocado growers, historically causing losses of $40 million annually (Coffey 1987, 1992; Ploetz 2013). The pathogen mostly infects the feeder roots, causing the roots to be blackened, brittle, or necrotic. Infected trees usually develop symptoms from pale green to yellow leaves, wilting, to heavy leaf fall and dieback, greatly reducing fruit yield. Trees eventually become leafless and die (Erwin and Ribeiro 1996).

P. cinnamomi is considered one of the most invasive pathogens in the world, because the pathogen has a wide host range, infecting over 3,000 hosts, including forest trees, such as eucalyptus, pine, and oak (Zentmyer 1980); ornamental plants, such as camellia, *Rhododendron*, and *Azalea*; and fruit crops, such as pineapple, peach, and highbush blueberry (Erwin and Ribeiro 1996; Shands et al. 2016). *P. cinnamomi* is a hemibiotrophic pathogen feeding initially from living host cells (biotrophic stage) and then switching to necrotrophy by killing the host cells and feeding from the nutrients released by them (necrotrophic phase) (Fawke et al. 2015; Hardham and Blackman 2018; Van den Berg et al. 2018). The entry into the plant is achieved by the adhesion of the motile zoospores to the host tissue, encystment, and germ tube formation. The germ tubes usually grow and penetrate the root surface via appressorium-like swelling structures and then plant tissue is rapidly colonized (Hardham 2005). During its biotrophic stage, *P.*

cinnamomi projects haustoria into the plant cells for the acquisition of nutrients and release of pathogen proteins (effectors) to aid the infection process in the host (Hardham and Blackman 2018; Huisman et al. 2015; Redondo et al. 2015; Van den Berg et al. 2018). This is followed by a necrotrophic stage characterized by host cell death, hyphal proliferation, and production of numerous sporangia (Evangelisti et al. 2017; Hardham 2005; Huisman et al. 2015). *P. cinnamomi* is heterothallic and both pathogen mating types (A1 and A2) are pathogenic, however, the A2 mating type is more invasive and is generally recognized as being the more aggressive (Kamoun et al. 2015). *P. cinnamomi* isolated from infected avocado trees almost exclusively consist of an A2 clonal population with no sexual reproduction evident. Even when both mating types are present in the population, diversity develops asexually in the form of clonal populations. Only one A1 *P. cinnamomi* isolate has been discovered on avocado in California, which appears to infect mainly alternate hosts such as camellia (Zentmyer and Guillemet 1981).

Once *P. cinnamomi* is introduced in a new avocado area, it cannot be eradicated, and for this reason, the ability to rapidly and accurately detect this pathogen, monitor its population, and differentiate their variants becomes more urgent to apply appropriate management strategies to reduce crop yield loss and pathogen spread. Despite the economic and ecological importance of *P. cinnamomi* worldwide, there are limited studies regarding: the genetic diversity of the pathogen population (Pagliaccia et al. 2013), the efficacy of novel Oomycota fungicides to manage avocado PRR, and the molecular and genetic basis of host-*P. cinnamomi* interactions (Meyer et al. 2016).

The first main objective of this dissertation was to assess the phenotype of avocado *P. cinnamomi* isolates representing the current clonal populations recovered in California. Recently, two different A2 clonal *P. cinnamomi* populations were found in California (Pagliaccia et al. 2013): the prevalent A2 clade I population and the more geographically specific A2 clade II population. The phenotypes of several avocado *P. cinnamomi* isolates corresponding to these A2 clades regarding *in vitro* mycelial growth rate, optimal growth temperature, sensitivity to registered Oomycota fungicides mefenoxam, potassium phosphite, and new compounds fluopicolide, and oxthiapiprolin, and virulence were evaluated. Finally, a detached leaf assay *P. cinnamomi* inoculation method using *Nicotiana benthamiana* was developed and validated to circumvent the difficulties associated with the avocado root inoculation method to assess the virulence of *P. cinnamomi* isolates.

Control strategies of avocado PRR are limited, and include: the use of resistant rootstocks, cultural practices, and chemical treatments. Commercially available root rot resistant rootstocks include Dusa®, Steddom, Thomas, Uzi, and Zentmyer (Bender et al. 2004). Among them, Dusa® is the industry standard currently in California, which enables growers to cultivate avocado in *P. cinnamomi* infested soil and maintain avocado production. Cultural practices include using certified disease-free nursery stock, keeping well-drained soil, cleaning tools and equipment, and applications of gypsum and mulch (Coffey 1987). Resistant rootstocks and cultural practices are effective ways to manage PRR, however, losses of avocado production to PRR are still substantial. Resistant rootstocks may also be overcome by aggressive *P. cinnamomi* populations (Belisle et al.

2018). Until rootstocks with strong levels of quantitative resistance are developed, there will be a need for chemical treatments to mitigate losses caused by PRR in avocado production.

At present, the only fungicides available to control PRR of avocado are phosphonate fungicides, e.g. potassium phosphite, and phenylamide fungicides, e.g. mefenoxam. Potassium phosphite injection is the preferred treatment by avocado growers due to the lack of reported resistance as well as the ability to apply this chemical as a fertilizer. Mefenoxam is used to a much less extent because of resistance development and the relatively higher cost. Both chemicals have been used for decades against *Phytophthora* spp. including *P. cinnamomi*. Aluminum tris-O-ethyl phosphonate was first introduced by Rhone-Poulenc Agrochimie Laboratories in 1977 with the product name Fosetyl-Al (Guest and Grant 1991). It was later discovered that potassium phosphite was the active ingredient and that alkyl phosphonates were degraded in the plant (Fenn and Coffey 1984). Potassium phosphite has been an effectively preventative and curative agent against avocado PRR since the early 1980s. However, there are only a limited number of reports on reduced *in vitro* sensitivities of a few *Phytophthora* spp., including: *P. capsici*, *P. cinnamomi*, *P. citrophthora*, *P. infestans*, *P. nicotianae*, and *P. syringae* (Adaskaveg et al. 2017; Cohen and Samoucha 1984; Veena et al. 2010; Wilkinson et al. 2001). Although the mode of action (MoA) of potassium phosphite is still unknown, its effectiveness is theorized to be the result of a number of interactions with both the pathogen and the plant host. Potassium phosphite directly inhibits *P. cinnamomi in vitro* at certain concentrations (Dobrowolski et al. 2008; Fenn and Coffey 1984; Ma and

McLeod 2014; Ouimette and Coffey 1989), but it has also been shown to be effective *in planta* at concentrations that were not inhibitory *in vitro* (Guest and Grant 1991). This suggests that potassium phosphite may prime plant defense responses. It is likely that the effectiveness of potassium phosphite is the result of a combination of both direct inhibition of the pathogen and an increase in the hosts natural defense response. Metalaxyl was developed by Ciba Geigy Ltd in 1977 and marketed as Ridomil (Guest and Grant 1991). Mefenoxam is an R-enantiomer of metalaxyl. Phenylamides affect the polymerase I complex of rRNA synthesis of oomycetes (Müller and Gisi 2012). Phenylamide resistance is the result of a mutation in a single incompletely dominant gene (Gisi and Sierotzki 2015), which has been found in *P. cinnamomi* as well as other *Phytophthora* spp., e.g. *P. infestans*, *P. citricola*, *P. megasperma*, and *P. nicotianae*, a few years after metalaxyl or mefenoxam was first applied (Coffey et al. 1984; Ferrin and Kabashima 1991; Gisi and Sierotzki 2015; Hwang and Benson 2005; Stack and Millar 1985). The risk of resistance development is considered as high (Gisi and Sierotzki 2015). Sensitivity to phenylamide fungicides of *Phytophthora* spp. has changed numerous times, especially for foliar pathogens such as *P. infestans*, due to the introduction of new clonal populations.

A few new Oomycota chemistries, including ethaboxam, fluopicolide, mandipropamid, and oxathiapiprolin, have been introduced to various oomycete diseases of different crops (Gisi and Sierotzki 2015; Jiang et al. 2015; Kim et al. 2004; Qu et al. 2016). All of these chemicals have unique MoAs that are also different from mefenoxam and potassium phosphite. Ethaboxam, a thiazole carboxamide, disrupts microtubule

organization in oomycetes such as *P. infestans*, *P. capsici*, *Plasmopara viticola*, and *Pseudoperonospora cubensis* (Uchida et al. 2005). Fluopicolide is a benzamide fungicide which delocalizes cytoskeleton-associated spectrin-like proteins (Jiang et al. 2015). It has proven effective against *P. capsici* on tomato as well as *P. nicotianae* on tobacco (Jiang et al. 2015; Qu et al. 2016). Mandipropamid is a carboxylic acid amide (CAA) fungicide, and its enzyme activity targets cellulose synthase in the pathogen (Gisi and Sierotzki 2015). It was shown to be effective against most *Phytophthora* spp. and *P. viticola* (Gisi and Sierotzki 2015; Lamberth et al. 2008). Oxathiapiprolin, a piperidinyl thiazole isoxazoline, targets the oxysterol binding protein of oomycetes (Miao et al. 2016a). This fungicide was found to be highly effective against numerous oomycete pathogens including *P. nicotianae*, *P. capsici*, *P. infestans*, and *P. citrophthora* (Bittner and Mila 2016; Gray et al. 2018; Miao et al. 2016b; Pasteris et al. 2016; Qu et al. 2016).

None of these new Oomycota fungicides have been registered to control PRR of avocado, so the second main objective of this dissertation was to evaluate the *in vitro* sensitivities of four new Oomycota-targeting fungicides (e.g. ethaboxam, fluopicolide, mandipropamid, and oxathiapiprolin) as well as currently registered fungicides (mefenoxam and potassium phosphite) to the *P. cinnamomi* population isolated in California avocado growing regions, and to evaluate the efficacy of these fungicides against avocado PRR in greenhouse studies.

The third main objective of this dissertation was to develop a *N. benthamiana*-*P. cinnamomi* model system to identify candidate genes associated with host resistance.

No plant resistance genes have been identified previously due to the lack of understanding of the genetic and molecular basis of plant-*P. cinnamomi* interactions. Identification of genes associated with host resistance to *P. cinnamomi* in avocado is a challenging task due to the limitations associated with the nature of tree crop biology that only one to three experiments can be completed annually. Currently, the avocado reference genome is not publicly available, and an efficient stable or transient avocado transformation system is still lacking (Pliego-Alfaro and Litz 2007). All of these factors make the application of functional genomics studies difficult in avocado. For this reason, we developed a new model system using the plant *N. benthamiana* to conduct these studies (Belisle et al. 2018). *N. benthamiana* has been widely used to study plant-*Phytophthora* interactions since it can be used for transient silencing and overexpression of genes (Bombarely et al. 2012; Evangelisti et al. 2017; Goodin et al. 2008; Helliwell et al. 2016) It was also recently applied to study plant-*P. palmivora* interactions (Evangelisti et al. 2017).

Zea mays, *Arabidopsis thaliana*, *Lupinus angustifolius*, *Castanea sativa* (chestnut), *Eucalyptus nitens*, *Lomandra longifolia*, and most recently *N. benthamiana* have all been investigated to better understand plant defense gene response to *P. cinnamomi* infection (Allardyce et al. 2013; Belisle et al. 2018; Eshraghi et al. 2014; Islam et al. 2017; Meyer et al. 2016; Rooks et al. 2008; Santos et al. 2017). Resistance to *P. cinnamomi* can be elucidated by comparing susceptible and resistant model plants. The gene expression between a susceptible and resistant variety of chestnut was compared and the defense gene expression was found to be significantly higher in the resistant

variety especially before inoculation. This increased basal defense to *P. cinnamomi* may contribute to the resistance of this variety (Santos et al. 2017).

Previous studies on avocado and model systems have provided important information on plant gene expression in response to *P. cinnamomi* infection. Several transcriptome studies have been completed on avocado. The initial study compared expressed sequence tags and 454 pyrosequencing results to identify defense related genes, which included: cytochrome P450, thaumatin, pathogenesis related protein 1 (PR1), metallothionein, MLO transmembrane protein encoding gene, and a universal stress protein (Mahomed and Van den Berg 2011). In a follow up study, 16 additional defense genes were described, including WRKY transcription factors, phenylalanine ammonia-lyase (PAL), and beta-glucanase (Reeksting et al. 2014). In Reeksting et al. (2016), up-regulated transcripts of interest included cytochrome P450 and germin-like protein (GLP). It has been stated that *P. cinnamomi* infection of model plants initiates different hormone signaling pathways compared to avocado infection (Reeksting et al. 2014). Currently, there seems to be some differences as well as many similarities in the gene expression of avocado compared to many model plants in response to *P. cinnamomi* infection. The differences in gene expression should be studied to better understand plant defense to *P. cinnamomi*.

This dissertation represents an integrated approach to study the molecular interaction of *P. cinnamomi* with its host and provide practical solutions to combat PRR of avocado in the field. This work will contribute in numerous ways to avocado growers in California. Identified variation in genotype and phenotype of prevalent *P. cinnamomi*

populations in California will greatly influence the development of resistant avocado rootstocks as well as efficacious chemical treatments. *P. cinnamomi* has also been isolated recently from blueberries, valued $123 million at production level in California. As avocado and blueberry fields are at times in close proximity, the presence of PRR in both crops could lead to changes in host resistance and virulence that would potentially be devastating to both industries. Ongoing drought and increased water salinity also necessitate this comprehensive approach to PRR management in California. Efficacious new fungicides on avocado plants will be registered for the treatment of avocado PRR. Candidate host resistance genes associated with and contributing to *P. cinnamomi* resistance will be targeted and used to implement Marker Assisted Screening (MAS) to develop new avocado rootstocks more resistant to the diverse population of *P. cinnamomi* in California.

REFERENCES

1. Adaskaveg, J. E., Förster, H., Hao, W., and Gray, M. 2017. Potassium phosphite resistance and new modes of action for managing Phytophthora diseases of citrus in the United States. Pages 205-210 in: Modern Fungicides and Antifungal Compounds. H. B. Deising, B. Fraaije, A. Mehl, E. C. Oerke, H. Sierotzki, and G. Stammler, eds. Vol. VIII. Deutsche Phytomedizinische Gesellschaft, Braunschweig, Germany.

2. Allardyce, J. A., Rookes, J. E., Hussain, H. I., and Cahill, D. M. 2013. Transcriptional profiling of *Zea mays* roots reveals roles for jasmonic acid and terpenoids in resistance against *Phytophthora cinnamomi*. Funct. Integr. Genomics 13:217-228.

3. Belisle R., McKee, B., Hao, W., Crowley, M., Arpaia, M. L., Miles, T. D., Adaskaveg, J. E., and Manosalva, P. 2018. Phenotypic characterization of genetically distinct *Phytophthora cinnamomi* isolates from avocado. Phytopathology 10.1094/PHYTO-09-17-0326-R.

4. Bender, G. S., Menge, J. A., and Arpaia., M. L. 2004. Avocado Rootstocks. Pages 45-53 in: Avocado Production in California: A Cultural Handbook for Growers. Book 1: Background Information, 2nd ed. G. S. Bender ed. University of California Cooperative Extension, San Diego County and The California Avocado Society, Oakland, CA.

5. Bittner, R. J., and Mila, A. L. 2016. Effects of oxathiapiprolin on *Phytophthora nicotianae*, the causal agent of black shank of tobacco. Crop Prot. 81:57-64.

6. Bombarely, A., Rosli, H., Verbalov, J., Moffett, P., Mueller, L. A., and Martin, G. B. 2012. A draft genome sequence of *Nicotiana benthamiana* to enhance molecular plant-microbe biology research. Mol. Plant Microbe Interact. 25:1523-1530.

7. Coffey, M. D. 1987. Phytophthora root rot of avocado - an integrated approach to control in California. California Avocado Society Yearbook 71:121-137.

8. Coffey, M. D. 1992. Phytophthora root rot of avocado. Pages 423-444 in: Plant Diseases of International Importantce. Volume III. Diseases of Fruit Crops. J. Kumar, H. S. Chaube, U. S. Singh, and A. N. Mukhopadhyay, eds. Prentice Hall, Englewood Cliffs, NJ.

9. Coffey, M. D., Klure, L. J., and Bower, L. A. 1984. Variability in sensitivity to metalaxyl of isolates of *Phytophthora cinnamomi* and *Phytophthora citricola*. Phytopathology 74:417-422.

10. Cohen, Y., and Samoucha, Y. 1984. Cross-resistance to 4 systemic fungicides in metalaxyl-resistant strains. Plant Dis. 68:137-139.

11. Dobrowolski, M. P., Shearer, B. L., Colquhoun, I. J., O'Brien, P. A., and Hardy, G. E. 2008. Selection for decreased sensitivity to phosphite in *Phytophthora cinnamomi* with prolonged use of fungicide. Plant Pathol. 57:928-936.

12. Erwin, D. C., and Ribeiro, O. K. 1996. Phytophthora Diseases Worldwide. APS Press, The American Phytopathological Society, St. Paul, MN.

13. Eshraghi, L., Anderson, J. P., Aryamanesh, N., McComb, J. A., Shearer, B., and Hardy, G. E. St. J. 2014. Suppression of the auxin response pathway enhances susceptibility to *Phytophthora cinnamomi* while phosphite-mediated resistance stimulates the auxin signaling pathway. BMC Plant Biol. 14:68.

14. Evangelisti, E., Gogleva, A., Hainaux, T., Doumane, M., Tulin, F., Quan, C., and Schornack, S. 2017. Time-resolved dual transcriptomics reveal early induced *Nicotiana benthamiana* root genes and conserved infection-promoting *Phytophthora palmivora* effectors. BMC Biol. 15:39.

15. Fawke, S., Doumane, M., and Schornack, S. 2015. Oomycete interactions with plants: infection strategies and resistance principles. Microbiol. Mol. Biol. Rev. 79:263-280.

16. Fenn, M. E., and Coffey, M. D. 1984. Studies on the *in vitro* and in vivo antifungal activity of fosetyl-A1 and phosphorous acid. Phytopathology 74:606-611.

17. Ferrin, D. M., and Kabashima, J. N. 1991. *In vitro* insensitivity to metalaxyl of isolates of *Phytophthora citricola* and *P. parasitica* from ornamental hosts in southern California. Plant Dis. 75:1041-1044.

18. Gisi, U., and Sierotzki, H. 2015. Oomycete fungicides: Phenylamides, Quinone Outside Inhibitors, and Carboxylic Acid Amides. Pages 145-174 in: Fungicide Resistance in Plant Pathogens. H. Ishii and D. W. Hollomon, eds. Springer, Tokyo, Japan.

19. Goodin, M. M., Zaitlin, D., Naidu, R. A., and Lommel, S. A. 2008. *Nicotiana benthamiana*: its history and future as a model for plant-pathogen interactions. Mol. Plant-Microbe Interact. 21:1015-1026.

20. Gray, M. A., Hao, W., Forster, H., and Adaskaveg, J. E. 2018. Baseline sensitivities of new fungicides and their toxicity to selected life stages of *Phytophthora* species from citrus in California. Plant Dis. 102:734-742.

21. Guest, D., and Grant, B. 1991. The complex action of phosphonates as antifungal agents. Biol Rev. Camb. Philos Soc. 66:159-187.

22. Hardham, A. R. 2005. *Phytophthora cinnamomi*. Mol. Plant Pathol. 6:589-604.

23. Hardham, A. and Blackman, L. 2018. *Phytophthora cinnamomi*. Mol. Plant Pathol. 19:260-285.

24. Helliwell, E. E., Vega-Arreguin, J., Shi, Z., Bailey, B., Xiao, S., Maximova, S. N., Tyler, B. M., and Guiltinan, M. J. 2016. Enhanced resistance in *Theobroma cacao* against oomycete and fungal pathogens by secretion of phosphatidylinositol-3-phosphate-binding proteins. Plant Biotechnol. J. 14:875-886.

25. Huisman, R., Bouwmeester, K., Brattinga, M., Govers, F., Bisseling, T., and Limpens, E. 2015. Haustorium formation in *Medicago truncatula* roots infected by *Phytophthora palmivora* does not involve the common endosymbiotic program shared by arbuscular mycorrhizal fungi and rhizobia. Mol. Plant Microbe Interact. 28:1271-1280.

26. Hwang, J., and Benson, D. M. 2005. Identification, mefenoxam sensitivity, and compatibility type of Phytophthora spp. attacking floriculture crops in North Carolina. Plant Dis. 89:185-190.

27. Islam, M.T., Hussain, H. I., Rookes, J.E., and Cahill, D.M. 2017. Transcriptome analysis, using RNA-Seq of *Lomandra longifolia* roots infected with *Phytophthora cinnamomi* reveals the complexity of the resistance response. Plant Biol. 20:130-142.

28. Jiang, L., Wang, H., Xu, H., Qiao, K., Xia, X. and Wang, K. 2015. Transportation behaviour of fluopicolide and its control effect against *Phytophthora capsici* in greenhouse tomatoes after soil application. Pest. Manag. Sci. 71: 1008-1014.

29. Kamoun, S., Furzer, O., Jones, J. D. G., Judelson, H. S., Ali, G. S., Dalio, R. J., Roy, S. G., Schena, L., Zambounis, A., Panabières, F., Cahill, D., Ruocco, M., Figueiredo, A., Chen, X. R., Hulvey, J., Stam, R., Lamour, K., Gijzen, M., Tyler, B. M., Grünwald, N. J., Mukhtar, M. S., Tomé, D. F., Tör, M., Van Den Ackerveken, G. , McDowell J., Daayf, F., Fry, W. E., Lindqvist-Kreuze. H., Meijer, H. J., Petre, B., Ristaino, J., Yoshida, K., Birch, P. R., and Govers, F. 2015. The Top 10 oomycete pathogens in molecular plant pathology. Mol. Plant

Pathol. 16:413-434.

30. Kim, D.-S., Chun, S.-J., Jeon, J.-J., Lee, S.-W., and Joe, G.-H. 2004. Synthesis and fungicidal activity of ethaboxam against Oomycetes. Pest. Manag. Sci. 60:1007-1012.

31. Lamberth, C., Jeanguenat, A., Cederbaum, F., De Mesmaeker, A., Zeller, M., Kempf, H. J., and Zeun, R. 2008. Multicomponent reactions in fungicide research: The discovery of mandipropamid. Bioorganic Med. Chem. 16:1531-1545.

32. Ma, J., and McLeod, A. 2014. *In vitro* sensitivity of South African *Phytophthora cinnamomi* to phosphite at different phosphate concentrations. South African Avocado Growers' Association Yearbook 37:79-84.

33. Mahomed, W. and Van den Berg, N. 2011. EST sequencing and gene expression profiling of defence-related genes from *Persea americana* infected with *Phytophthora cinnamomi***.** BMC Plant Biol. 11:167.

34. Meyer, F. E., Shuey, L. S., Ramsuchit, S., Mamni, T., Berger, D. K., Van den berg, N., Myburg, A. A., and Naidoo, S. 2016. Dual RNA-sequencing of *Eucalyptus nitens* during *Phytophthora cinnamomi* challenge reveals pathogen and host factors influencing compatibility. Front. Plant Sci. 7:191.

35. Miao, J., Cai, M., Dong, X., Liu, L., Lin, D., Zhang, C., Pang, Z., and Liu, X. 2016a. Resistance assessment for oxathiapiprolin in *Phytophthora capsici* and the detection of a point mutation (G769W) in PcORP1 that confers resistance. Front Microbiol. 7:615.

36. Miao, J, Dong, X, Lin, D., Wang, Q., Liu, P, Chen, F., Du, Y., and Liu, X. 2016b. Activity of the novel fungicide oxathiapiprolin against plant-pathogenic oomycetes. Pest. Manag. Sci. 72:1572-1577.

37. Müller, U., and Gisi, U. 2012. Newest Aspects of Nucleic Acid Synthesis Inhibitors: Metalaxyl-M. Pages 901-908 in: Modern Crop Protection Compounds, 2nd ed. W. Krämer, U. Schirmer, P. Jeschke, and M. Witschel eds. Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim, Germany.

38. Ouimette, D. G., and Coffey, M. D. 1989. Comparative antifungal activity of four phosphonate compounds against isolates of nine *Phytophthora* species. Phytopathology 79:761-767.

39. Pagliaccia, D., Pond, E., McKee, B., and Douhan, G. W. 2013. Population genetic structure of *Phytophthora cinnamomi* associated with avocado in California and

the discovery of a potentially recent introduction of a new clonal lineage. Phytopathology 103:91-97.

40. Pasteris, R. J., Hanagan, M. A., Bisaha, J. J., Finkelstein, B. L., Hoffman, L. E., Gregory, V., Andreassi, J. L., Sweigard, J. A., Klyashchitsky, B. A., Henry, Y. T., and Berger, R. A. 2016. Discovery of oxathiapiprolin, a new oomycete fungicide that targets an oxysterol binding protein. Bioorg. Med. Chem. 24:354-361.

41. Pliego-Alfaro, F. and Litz, R. E. 2007. Biotechnology and avocado improvement. California Avocado Society Yearbook. 90:149-167.

42. Ploetz, R. C. 2013. Phytophthora root rot of avocado. Pages 197-203 in: *Phytophthora*: A Global Perspective. K Lamour ed. CABI Plant Protection Series, Vol. 2. CABI, Boston, MA.

43. Qu, T., Shao, Y., Csinos, A. S., and Ji, P. 2016. Sensitivity of *Phytophthora nicotianae* from tobacco to fluopicolide, mandipropamid, and oxathiapiprolin. Plant Dis. 100:2119-2125.

44. Redondo, M., Perez-Sierra, A.; Abad-Campos, P., Torres, L., Solla, A., Reig-Armiñana, and Garcia-Breijo, F. 2015. Histology of Quercus ilex roots during infection by *Phytophthora cinnamomi*. Trees 29:1943-1957.

45. Reeksting, B. J., Coetzer, N., Mahomed, W., Engelbrecht, J., and van den Berg, N. 2014. De novo sequencing, assembly, and analysis of the root transcriptome of *Persea americana* (Mill.) in response to *Phytophthora cinnamomi* and flooding. PLoS ONE 9:e86399.

46. Reeksting, B. J., Coetzer, Olivier, N. A., and van den Berg, N. 2016. Transcriptome responses of an ungrafted Phytophthora root rot tolerant avocado (*Persea americana*) rootstock to flooding and *Phytophthora cinnamomi*. BMC Plant Biol. 16:205.

47. Rookes, J. E., Wright, M. L., and Cahill, D. M. 2008. Elucidation of defense responses and signaling pathways induced in *Arabidopsis thaliana* following challenge with *Phytophthora cinnamomi*. Physiol. Mol. Plant Pathol. 72:151-161.

48. Santos, C., Duarte, S., Tedesco, S., Fevereiro, P., and Costa, R. L. 2017. Expression profiling of castanea genes during resistant and susceptible interactions with the oomycete pathogen Phytophthora cinnamomi reveal possible mechanisms of immunity. Front. Plant Sci. 8:515

49. Shands, A. C., Yamagata, J. S., Wright, A. F., and Miles, T. D. 2016. First report of *Phytophthora cinnamomi* causing root rot of Southern highbush blueberry in California. Plant Dis. 100:537.

50. Stack, J., and Millar, R. 1985. Isolation and characterization of a metalaxyl-insensitive isolate of *Phytophthora megasperma* f. sp. *medicaginis*. Phytopathology 75:1387-1392.

51. Uchida, M., Roberson, R. W., Chun, S.-J. and Kim, D.-S. 2005. In vivo effects of the fungicide ethaboxam on microtubule integrity in *Phytophthora infestans*. Pest. Manag. Sci. 61:787-792.

52. Van den Berg, N., Christie, J. B., Aveling, T. A. S., and Engelbrecht, J. 2018. Callose and β-1-3 glucanase inhibit *Phytophthora cinnamomi* in a resistant avocado rootstock. Plant Pathol. 67:1150-1160.

53. Veena, S. S., Anandaraj, M., and Sarma, Y. R. 2010. Variability in the sensitivity of *Phytophthora capsici* isolates to potassium phosphonate. Indian Phytopathol. 63:71-75.

54. Wilkinson, C. J., Shearer, B. L., Jackson, T. J., and Hardy, G. E. S. 2001. Variation in sensitivity of Western Australian isolates of *Phytophthora cinnamomi* to phosphite *in vitro*. Plant Pathol. 50:83-89.

55. Zentmyer, G. A. 1980. *Phytophthora cinnamomi* and the diseases it causes. Monograph No. 10. The American Phytopathological Society, St. Paul, MN.

56. Zentmyer, G. A., and Guillemet, F. B. 1981. Evidence for strains of *Phytophthora cinnamomi*. Plant Dis. 65:475-477.

CHAPTER II. PHENOTYPIC CHARACTERIZATION OF GENETICALLY DISTINCT *PHYTOPHTHORA CINNAMOMI* ISOLATES FROM AVOCADO

Belisle, R. J., McKee, B., Hao, W., Crowley, M., Arpaia, M. L., Miles, T. D., Adaskaveg, J. E., and Manosalva, P. 2018. Phenotypic characterization of genetically distinct *Phytophthora cinnamomi* isolates from avocado. Phytopathology (*in press*). https://doi.org/10.1094/PHYTO-09-17-0326-R

ABSTRACT

Phytophthora cinnamomi, the causal agent of Phytophthora Root Rot (PRR), is the most destructive disease of avocado worldwide. A previous study identified two genetically distinct clades of A2 mating type avocado isolates in California, however the phenotypic variation among them was not assessed. This study described the phenotype of a subset of isolates from these groups regarding growth rate, growth temperature, virulence, and fungicide sensitivity. Isolates corresponding to the A2 clade I group exhibited higher mycelial growth rate and sensitivity to higher temperatures than other isolates. Among the fungicides tested, potassium phosphite had the highest 50% effective concentration for mycelial growth inhibition and oxathiapiprolin had the lowest. Mycelial growth rate and potassium phosphite sensitivity phenotypes correlate with specific group of isolates suggesting that these traits could be a group characteristic. Moreover, isolates that are more virulent in avocado and less sensitive to potassium phosphite were

identified. A detached leaf *P. cinnamomi* inoculation method using *Nicotiana benthamiana* was developed and validated providing an alternative method for assessing the virulence of a large number of isolates. This information will help avocado PRR management and assist breeding programs for the selection of rootstocks resistant against a more diverse pathogen population.

INTRODUCTION

The oomycete pathogen *Phytophthora cinnamomi* Rands, causal agent of Phytophthora root rot (PRR), is the most destructive disease of avocado worldwide (Erwin and Ribeiro 1996). In California, avocado PRR affects 60-75% of avocado growers who lose approximately $40 million annually (Ploetz 2013). This globally distributed oomycete is called "the biological bulldozer" for its capacity to infect over 3000 plant species causing devastating impacts in natural ecosystems, forestry, agriculture, and the nursery industry (Hardham 2005; Hardham and Blackman 2018; Kamoun et al. 2015). The economic impact due to *P. cinnamomi* infestation is evident in the forest and food industry, affecting eucalyptus, pine, oak (Zentmyer 1980), and other fruit crops such as pineapple, peach, and highbush blueberry (Shands et al. 2016). Losses include not only decreases in crop yield and product value, but also large amounts of money spent annually on control measures. There are no effective means to eradicate *P. cinnamomi* from infested areas as it survives in moist soil or dead plant material as chlamydospores for long periods under adverse conditions (Hardham and Blackman 2018; Reitmann et al. 2017; Zentmyer and Mircetich 1966). Several PRR control strategies have been found to reduce the impact of this invasive pathogen including the use of chemical treatment (phosphonate-based and mefenoxam), tolerant plants, and management practices (e.g. proper diagnostics, mulching, and proper irrigation).

P. cinnamomi is a hemibiotrophic pathogen feeding initially from living host cells (biotrophic stage) and then switching to necrotrophy by killing the host cells and feeding

from the nutrients released by them (necrotrophic phase) (Fawke et al. 2015; Hardham and Blackman 2018; Van den Berg et al. 2018). The entry into the plant is achieved by the adhesion of the motile zoospores to the host tissue, encystment, and germ tube formation. The germ tubes usually grow and penetrate the root surface via appressorium-like swelling structures and then plant tissue is rapidly colonized (Hardham 2005). During its biotrophic stage, *P. cinnamomi* projects haustoria into the plant cells for the acquisition of nutrients and release of pathogen proteins (effectors) to aid the infection process in the host (Hardham and Blackman 2018; Huisman et al. 2015; Redondo et al. 2015; Van den Berg et al. 2018). This is followed by a necrotrophic stage characterized by host cell death, hyphal proliferation, and production of numerous sporangia (Evangelisti et al. 2017; Hardham 2005; Huisman et al. 2015). Currently, the molecular and genetic basis of *P. cinnamomi* pathogenicity, virulence, and plant immunity against this pathogen are largely unknown due to limitations associated with tree crop biology and the lack of tools available for functional studies in tree crops such as avocado (Pliego-Alfaro and Litz 2007). *Arabidopsis* and lupin have been used as model systems to study *P. cinnamomi*-plant interactions (Allardyce et al. 2013; Esharghi et al. 2011; Rookes et al. 2008). The model plant, *Nicotiana benthamiana* (Bombarely et al. 2012), has been widely used to study the pathogenicity and virulence of similar broad range and root Phytophthora pathogens such as *P. capsici* (Chen et al. 2013; Vega-Arreguin et al. 2014), *P. palmivora* (Ekchaweng et al. 2017; Evangelisti et al. 2017; Goodin et al. 2008), and *P. parasitica* (Dalio et al. 2018; Robin and Guest 1994). Moreover, several studies using model plants, crops, and tree crops to study pathogenicity, virulence, and fungicide

efficacy of PRR pathogens such as *P. sojae*, *P. capsici*, *P. parasitica*, *P. palmivora*, *P. cinnamomi*, and *P. ramorum* have been done using detached-leaf assays (Denman et al. 2005; Dong et al. 2015; Ekchaweng et al. 2017; Esharghi et al. 2011; Helliwell et al. 2016; Robin and Guest 1994; Vega-Arreguin et al. 2014).

Phosphite is the most widely used chemical control method for managing PRR caused by several *Phytophthora* spp. including *P. cinnamomi* (Dobrowolski et al. 2008; Ma and McLeod 2014). Phosphorous acid dissociates to form the phosphonate ion (HPO_3^{2-}), also called phosphite. Phosphorous acid and its ionized compounds are often referred to as phosphonate or phosphonite. The specific mode of action of potassium phosphite is largely unknown, however appears to involve both a direct and an indirect effect on the pathogen (Eshraghi et al. 2014a; Guest and Grant 1991; McGrath 2004). Several studies have assessed the *in vitro* sensitivity of *P. cinnamomi* to phosphite using mycelial radial growth inhibition in solid and liquid media to identify sensitive and tolerant isolates (Dobrowolski et al. 2008; Fenn and Coffey 1984; Ma and McLeod 2014; Ouimette and Coffey 1989). In California, avocado growers heavily rely on the use of phosphite products to control *P. cinnamomi*, however the phosphite sensitivity of California avocado isolates is largely unknown.

In addition to phosphite, phenylamide fungicides such as metalaxyl and mefenoxam are also used for managing diseases caused by oomycetes including *P. cinnamomi* (Benson and Grand 2000; Erwin and Ribeiro 1996; Hu et al. 2010; Schwinn and Staub 1995). Resistance to metalaxyl has developed in *P. capsici*, *P. infestans*, and *P. nicotianae* (Lamour and Hausbeck 2001; Schwinn and Staub 1995; Shew 1985).

Phenylamides usually do not inhibit germination of sporangia or encysted zoospores as effectively as they do mycelial growth (Staub and Young 1980). Consequently, inhibition of mycelial growth *in vitro* has been used as the primary method of determining the sensitivity to these fungicides among isolates of *Phytophthora* spp. (Benson and Grand 2000; Coffey 1984 and Bower). The need for new oomycete-targeted fungicides to control diseases caused by these pathogens especially those that have developed resistance to phenylamide fungicides has resulted in the development of several new chemicals with varying modes of action such as fluopicolide (Presidio; Valent USA, Walnut Creek, CA) and oxathiapiprolin (Orondis; Syngenta Crop Protection, Greensboro, NC). Fluopicolide is a pyridinylmethyl-benzamide (acylpicolide) fungicide that disrupts cell division and mitosis by acting on spectrin-like proteins (Briggs et al. 2006). This fungicide is effective to control diseases caused by *P. capsici* (Keinath and Kousik 2011) and *P. infestans* (Saville et al. 2015). Oxathiapiprolin is the first of the new piperidinyl thiazole isoxazoline class fungicides discovered and developed by DuPont Co. in 2007. The molecular target of oxathiapiprolin is the oxysterol binding protein (OSBP) (Andreassi et al. 2013). This new fungicide exhibits strong inhibitory activity against a range of agriculturally important plant-pathogenic oomycetes including *P. capsici*, *P. infestans*, *P. sojae*, *Peronospora belbahrii*, and *Pythium ultimun* (Miao et al. 2016; Pasteris et al. 2016; Patel et al. 2015). However, its inhibitory activity against *P. cinnamomi* has not been tested.

P. cinnamomi is a heterothallic species that requires the presence of both A1 and A2 mating types to undergo sexual reproduction. Despite that both mating types are

pathogenic (Kamoun et al. 2015), avocado PRR disease in California is mainly associated with A2 mating type isolates (Pagliaccia et al. 2013). Previous *P. cinnamomi* population studies have revealed low levels of genotypic and phenotypic variation (Beaulieu et al. 2017; Dobrowolski et al. 2003; Duan et al. 2008; Eggers et al. 2012; Engelbrecht et al. 2017; Herrera and Perez-Jimenez 1995; Hu et al. 2010; Linde et al. 1997, 1999; Ma and McLeod 2014; Martin and Coffey 2012; Pagliaccia et al. 2013; Zentmyer et al. 1976, Zentmyer 1980) among isolates from different mating types, origin, isolation source, and host plants, however, only a few were conducted or have included isolates from avocado (Engelbrecht et al. 2017; Herrera and Perez-Jimenez 1995; Linde et al. 1999; Ma and McLeod 2014; Martin and Coffey 2012; Pagliaccia et al. 2013; Zentmyer et al. 1976, Zentmyer 1980). These studies described the existence of three clonal lineages for *P. cinnamomi*, one corresponding to the A1 mating type isolates and two different clonal lineages for the A2 mating type isolates (A2 type 1 and A2 type 2) (Dobrowolski et al. 2003; Eggers et al. 2012). Pagliaccia et al. (2013) conducted the first study to assess the genetic diversity of *P. cinnamomi* isolates from avocado in California and also found two genetically distinct clades of A2 mating type isolates (A2 clade I and clade II). The A2 clade II consisted of isolates with unique genotypes collected only in Southern California. Interestingly, the authors in this study included isolates from *P. cinnamomi* previously identified as belonging to the A2 type 1 and A2 type 2 described by Dobrowolski et al. (2003), however these isolates clustered within the A2 clade I group, suggesting that the A2 clade II group identified in Paglaccia et al. (2013) in California could be another clonal lineage.

No studies have been conducted to assess the phenotype of avocado isolates corresponding to these genetically distinct A2 groups identified in California by Paglaccia et al. (2013), therefore, the objectives of this study were to i) assess the phenotype of several avocado isolates corresponding to these A2 clades regarding *in vitro* mycelial growth rate, optimal growth temperature, sensitivity to potassium phosphite and mefenoxam, and virulence, ii) test the sensitivity of avocado isolates to fluopicolide and oxathiapiprolin as alternative chemistries for controlling avocado PRR in California, and iii) develop and validate a detached leaf assay inoculation method using *N. benthamiana* to circumvent the difficulties associated with the avocado whole plant root inoculation method to assess the virulence of *P. cinnamomi* isolates. This information will help to design appropriate measures for managing avocado PRR in California and implement efficient and reliable screening methods towards the selection and development of new *P. cinnamomi* resistant avocado rootstocks effective against a more diverse pathogen population.

MATERIALS AND METHODS

***Phytophthora cinnamomi* isolates.** A total of twelve *P. cinnamomi* isolates associated with avocado PRR were used in this study, including six isolates from Northern California corresponding to the A2 clade I group, four isolates corresponding to the unique A2 clade II subpopulation from Southern California, and two isolates collected from the same Southern California region from where the A2 clade II isolates

were collected (Supplementary Table 2.1). A2 clade I isolates from this study represent five (N2, N3, N4, N6, and N7) of the ten total genotypes identified by Paglaccia et al. (2013). Isolates representing the A2 clade II group covered three (S3, S5, and S6) from the six unique genotypes identified in Southern California. All the isolates in this study were obtained from a *P. cinnamomi* small collection at the UCR Avocado Rootstock Breeding Program. Isolates were maintained as water agar plugs (Boesewinkel 1976). Agar plugs were removed one at the time as needed for each experiment and plated on 10% clarified cV8A agar (10 g of $CaCO_3$ per 1 liter of V8 juice spun down at 4000 rpm for 20 min) to resume growth. To ensure that isolates in the collection are free of contamination and avoid any problems with mix samples or not appropriate maintenance of the collection, we sequenced all the isolates using ITS and *COX1* spacer regions. Mycelial DNA was extracted using the Qiagen DNeasy plant mini kit (Qiagen, Germantown, MD). Universal primers ITS1 and ITS4 were used for the ITS region, and primers OOM and FM85 were used for the *COX1* spacer region, both sets of primers are described in Kroon et al. (2004). Each 25-µl PCR reaction contained 2 µl of DNA (50 ng/µl), 2.5 µl of 10x PCR buffer (New England Biolabs, Ipswich, MA), 200 µM dNTPs, 0.4 µM of each primer, and 1.25 units of Taq DNA polymerase (New England Biolabs, Ipswich, MA). PCR reactions were performed using a Programmed Thermal Controller (PTC-100; MJ Research, Inc., Watertown, MA) with conditions as follows: 95°C for 5 min; followed by 35 cycles at 95°C for 1 min, 50°C for 1 min, and 68°C for 1 min; and a final extension at 68°C for 10 min. PCR products were confirmed on 1% certified molecular biology agarose gel (Bio-Rad Laboratories), stained with ethidium bromide

(0.05% v/v), and visualized under UV light using a Universal Hood UV Light Table (75s/00124 With Camera Model Rs170s; Bio-Rad Laboratories). PCR products were treated with Zymo DNA Clean and Concentrator (Zymo Research Corp, Irvine, CA) according to the manufacturer's instructions to remove excess primers and nucleotides before submitting the samples for Sanger sequencing. DNA sequencing was conducted in both directions with the same primers used for amplification at the UCR Genomics Core facility. Contiguous sequences were generated which were then subjected to a BLASTn search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to determine the highest maximum identity to the sequence of the type isolates in GenBank.

Plant material. Two avocado rootstocks were selected based on their *P. cinnamomi* resistance phenotype. Clonal rootstocks corresponding to the moderate resistant Dusa® (industry standard) and the susceptible PS.54 (Westfalia Technological Services, Tzaneen, South Africa) were obtained from Brokaw Nursery. The 6-month-old clonally propagated plants were removed from their bags and transplanted into pots after the nurse seed was removed. Plants were grown in a greenhouse with an average maximum temperature range of 25-28°C at 40-50% relative humidity. Plants were fertilized twice a week and watered every day. *Nicotiana benthamiana* seeds were germinated in trays and transferred to individual pots 2 weeks post germination. Plants were grown at 22°C with 16 h of light and 8 h dark cycles at 40-50% relative humidity.

Effect of temperature and medium on *P. cinnamomi* mycelial growth rate. Temperature is one of the most significant environmental factors affecting growth, reproduction, and pathogenesis of fungi and oomycetes pathogens (Zentmyer et al. 1976).

The temperatures tested in this study were selected based on the optimal growth temperature range reported for *P. cinnamomi* 20°C to 30°C (Phillips and Weste 1985; Zentmyer et al. 1976). Mycelial growth rate (mm/day) was determined at 22°C, 25°C, and 28°C on 10% cV8A following the methodology described by Chen and Zentmyer (1970). Mycelial growth rate on 10% cV8A at 22°C was also compared to the growth rate on corn meal agar (CMA) (Eckert and Tsao 1962) (Becton Dickinson, Sparks, MD). Seven-mm-diameter agar plugs were cut from the margin of a 4-day-old colony of *P. cinnamomi* cV8A plates, transferred to their corresponding media plates, and incubated in the dark for 4 days. Colony diameter was measured at 2 and 4 days after plug transfer.

***In vitro* fungicide sensitivity.** The effective concentration of potassium phosphite at which 50% of the mycelial growth of each isolate was inhibited (EC_{50} value) was determined using the traditional agar dilution method as described in Adaskaveg et al. (2015). Potassium phosphite (ProPhyt, 34.3% phosphorous acid; Helena Chemical, Collierville, TN) was added to 10% cV8A to obtain final concentrations of 5, 25, and 100 μg/ml. Mycelial agar plugs (6 mm in diameter) from 6-day old cultures of *P cinnamomi* were placed at the center of the plates containing the corresponding chemical concentration. Three replicates were done per each treatment. After incubation for 3 days in the dark at 22°C, radial colony growth was measured. Colony growth inhibition of each isolate in the presence of potassium phosphite was then calculated as compared with their corresponding control plates without potassium phosphite. This experiment was conducted at least twice. Calculation of the EC_{50} values for potassium phosphite of each isolate was determined as described in Adaskaveg et al. (2015). Briefly, the EC_{50} values

were determined by fitting regression lines of the natural log-transformed phosphite concentration against the logit-transformed percent inhibition as compared with the control for each isolate. Regression equations were then solved for concentration at 50% inhibition using Microsoft Excel (version 15.41).

The EC_{50} values for mefenoxam (Ridomil Gold SL; Syngenta Crop Protection), fluopicolide (Presidio; Valent USA), and oxathiapiprolin (Orondis; Syngenta Crop Protection), currently used to control diseases caused by oomycete pathogens, were determined using the spiral gradient dilution method described by Förster et al. (2004). Mefenoxam, oxathiapiprolin, and fluopicolide were dissolved in sterile water to make the corresponding stock solutions at 50, 5, and 100 µg/ml, respectively. Each fungicide was radially applied to a 15-cm 10% cV8A plate using a spiral plater (Autoplate 4000; Spiral Biotech, Norwood, MA). Isolates were grown for 7 days in the dark at 22°C on 10% cV8A media. Cultures were then cut into strips and placed between sterile hydrophilic cellophane strips (5.5 cm × 0.5 cm; du Pont de Nemours, Wilmington, DE) on new 10% cV8A plates. Plates were incubated for another 7 days in the dark at 22°C or until the mycelium had evenly covered the strips. Mycelium-covered cellophane strips were then radially applied across the fungicide concentration gradient on the spiral gradient dilution fungicide plate. The ranges of concentrations used were between 0.0025 to 0.6837 µg/ml (1:274 gradient dilution), 0.0038 to 1.4054 µg/ml (1:370), and 0.0002 to 0.0572 µg/ml (1:286) for mefenoxam, fluopicolide, and oxathiapiprolin, respectively. For each isolate, duplicated strips were placed at the opposite location on each spiral gradient dilution fungicide plate (two replications). Controls consisted of cV8A plates spirally plated with

water, to which cellophane strips were placed as described above. Plates were incubated in the dark at 22°C. Two independent experiments were conducted. Two days after incubation, the mycelial growth of each isolate in each of the control plates was measured. The location where mycelial growth was inhibited by 50%, compared with growth on the control plates, was determined. The distance between this location and the center of the plate was measured. This distance was used to determine the local fungicide concentration on the agar at the location of 50% inhibition using the SGE software (EC_{50} value). The EC_{50} values for each fungicide were calculated using the spiral gradient endpoint (SGE) software (version 1.3; Spiral Biotech, Norwood, MA) as described in Förster et al. (2004).

Avocado rootstock inoculation. Nine-month-old avocado rootstocks liners were inoculated with *P. cinnamomi* colonized millet seed. Millet inoculum was prepared according to the method of Drenth and Sendall (2001). Briefly, 175 ml distilled water was added to 144 g of millet seed. Excess water was poured off and moistened millet was autoclaved in a 250 ml volumetric flask. Autoclaving was repeated the following day. For each isolate, ten plugs of 6-mm-diameter were cut from the margin of a 4-day-old colony of *P. cinnamomi* plate and were added to the flask containing the sterilized millet seeds. Inoculated millet was incubated in the dark at 25°C for 21 days. The millet was then harvested, weighed, and homogenized in a plastic bag. A total of 4.4 g of inoculum per plant was used for inoculation by placing the inoculated millet around the root collar. Combination isolate inoculum was produced by thoroughly mixing equal amounts of each corresponding isolate inoculated millet seeds together. Dusa® and PS.54 ungrafted

clonal rootstocks were inoculated with individual isolates, a combination of Northern California isolates (North mix), a combination of Southern California isolates (South mix), and a combination of all isolates (All mix). Control plants were treated with uninoculated millet seeds that were sterilized and prepared in the same manner as the inoculated millet seeds. The experiment was conducted in a greenhouse using a randomized complete block design with five replicates per treatment. The average maximum temperature in the greenhouse was 28°C with an average minimum temperature of 14°C. To confirm that plants were successfully infected, roots samples were excised from harvested plants and 10 to 20 segments per plant were surface sterilized in 70% ethanol and plated onto the PARPH-V8 *Phytophthora*-selective media (Delvocid Pimaricin 5 μg/ml, Sodium Ampicillin 250 μg/ml, Rifamycin 10 μg/ml, Terraclor [PCNB] 50 μg/ml, and Tachigaren [70% hymexazol] 50 μg/ml. Plates were incubated at 22°C for 2 to 3 days in the dark. *Phytophthora* infected root pieces were counted on each plate and the presence of *P. cinnamomi* was confirmed based on morphological characteristics. Same procedure was done with the roots of the control plants inoculated with the uninoculated millet, no growth on the roots was observed.

***Nicotiana benthamiana* detached leaf inoculations.** Five-week-old *N. benthamiana* were used for detached leaf inoculations as described by Manosalva et al. (2015) using zoospore suspensions. *Z*oospores suspensions were produced following the protocol described by Londsdale et al. (1988). Briefly, 10 (7mm) plugs from 6-day-old cultures grown on 10% cV8 media were placed on sterile Miracloth (EMD Millipore Corp., Billerica, MA) previously placed on a new 10% cV8 plate. Plugs were incubated

for 6 days at 22°C in the dark, the Miracloth was then removed from the plate and placed in a flask with 100 mL of cV8 broth. After 20 hours of incubation at 22°C in the dark shaking at 160 rpm, the Miracloth was rinsed 3 times with a salt solution (Londsdale et al. 1988) then incubated for an additional 18 hours at 22°C in the dark shaking at 160 rpm. The Miracloth was then rinsed with 100 mL of 18°C water and incubated in 50 mL of 18°C water for 1.5 hours to induce zoospore release. The abaxial leaf surface of *N. benthamiana* leaves were drop inoculated using 20 µl of a zoospore suspension adjusted to 1×10^4 zoospores/ml using a hemocytometer (Bright-Line, Horsham, PA). The inoculated leaves were placed in petri dishes containing 1.5% water agar and incubated in a precision plant growth incubator (Thermo Scientific, Waltham, MA) under 18 h dark and 6 h light cycles at 25°C. Lesions were measured at 3 days post inoculation.

Trypan blue staining. Leaves of *N. benthamiana* were stained following the protocol described in Manosalva et al. (2015) at 3, 6, 12, 24, 48, 72, and 96 h post-inoculation to assess the progression of *P. cinnamomi* infection. Briefly, leaf tissue was boiled for 5 min in a 1:1 mixture of ethanol and staining solution (10 ml lactic acid, 10 ml glycerol, 10 ml phenol, and 10 mg trypan blue dissolved in 10 ml of Milli Q water). Leaf samples were mounted using 50% glycerol (v/v) and imaged using a Keyence BZ-X700 fluorescence microscope (Keyence Corporation, Itasca, IL).

***Phytophthora cinnamomi* DNA quantification using TaqMan real-time PCR *in planta*.** Four leaf discs (10 mm) excised from the inoculated area of *N. benthamiana* leaves at all the time points indicated above were used for genomic DNA extractions using a modified 2% CTAB cetyl-trymethyl-ammonium bromide (Acros Organics, Fair

Lawn, NJ) protocol (Brunner et al. 2001). *P. cinnamomi* DNA was quantified using the TaqMan real-time PCR protocol described by Miles et al. (2017). For the *atp9-nad9* marker system used in this study, PhyG_ATP9_2FTail (5′-AATAAATCATAACCTTCTTTACAACAAGAATTAATG-3′), PhyG-R6_Tail (5′-AATAAATCATAAATACATAATTCATTTTTATA-3′) and Bandaid primer (5′-AATAAATCATAAATACATAATTCRTTTTTRTA-3′) served as amplification primers and a *cox* primer set FMPl2b (5′-GCGTGGACCTGGAATGACTA-3′) and FMPl3b (5′-AGGTTGTATTAAAGTTTCGATCG-3′) served as the internal control (Bilodeau et al. 2014; Miles et al. 2017). The "bandaid" primer is an additional reverse primer used to increase the amplification sensitivity for *P. cinnamomi* isolates containing single nucleotide polymorphisms (SNPs) in the reverse *nad9* primer (PhyG-R6_Tail) (Miles et al. 2017). Amplification reactions were observed using TaqMan based probes including the *Phytophthora* genus specific ATP9_PhyG2_probeR (5'-[Fam]AAAGCCATCATT-AAACARAATAAAGC[BHQ1]-3'), Plant CAL-Red probe (5'-[CALFluorRed610]CTT-TTATTATCACTTCCGGTACTGGCAGG[BHQ-2]-3') and the *P. cinnamomi* Pcinn_nad9sp_probe1 (5'-[Quasar670]AAGAAATATTTAGTTTATTAATATATAAT-ATAACT[BHQ2]-3') (Miles et al. 2017). TaqMan real-time PCR was performed in a CFX96 Real-Time PCR system (Bio-Rad Laboratories, Hercules, CA). Amplification reaction contained 12.5µL of 2.5x PerfeCTa Tough Mix (Quantabio, Beverly, MA), 3µl of 50 mM Mg, 500 nM each of the *nad9* primers, 8 nM Bandaid primer, 12.5 nM each of *cox* primers, 50 nM each of the Phytophthora probes, 10 nM of the plant probe, and 1µl of the DNA template (50 ng/µl) in 25 µl total volume reaction. Thermal cycling consisted

of 95°C for 2 min, 50 cycles at 95°C for 15 s and 1 min and 30 s at 57°C. Absolute quantification for the *atp9-nad9* marker data was calculated based on a serial dilution standard curve plot constructed using different known concentrations of DNA from *P. cinnamomi* isolate N-2113. Two technical replications were performed for each of the three biological replications for each time point.

Statistical analysis. Colony growth rate, fungicide sensitivity, lesion size in *N. benthamiana*, and TaqMan qPCR quantification results were analyzed using one-way ANOVA and the differences between means were compared using Fisher's least significant difference (LSD) test. All these analyses were performed in R (3.1.3) (R Core Team 2015) using the agricolae package (Mendiburu 2015). Results were considered significant at $P \leq 0.05$. All assays had at least three replicates for each isolate and each experiment was repeated at least three times. Virulence test in avocado was analyzed using one-way random effects ANOVA and the differences between means were compared using Tukey's honest significant difference (HSD) test ($P \leq 0.05$). These analyses were conducted using JMP Pro 13 software (SAS Institute Inc., Cary, NC). Five replicates were used for each rootstock and the experiment was repeated three times.

RESULTS

Effect of temperature on mycelial growth rate. Mycelial growth rates using cV8A nutrient media varied among isolates at 22°C, 25°C and 28°C within and between groups (Table 2.1). None of the 12 isolates tested in this study differed significantly in

colony morphology (*data not shown*). At 22°C all A2 clade I isolates collected from Northern California (Northern, N) had significantly higher growth rates than isolates collected from Southern California (Southern, S). The mycelia growth rate of the Northern group of isolates at this temperature ranged from 8.4 ± 0.08 to 6.7 ± 0.06 mm/day. In this group, N-2114 has the highest growth rate among all isolates tested in this study at 22℃ and 25℃ but not at 28℃ where the significant differences on growth rates among all isolates within and between groups were reduced (Table 2.1). The growth rate of the Southern isolates tested ranged from 4.5 ± 0.12 to 5.88 ± 0.05 mm/day at 22℃. Within this group, S-2117 and S-2120 exhibited the highest growth rates (5.6 ± 0.3 and 5.9 ± 0.05 mm/day, respectively) (Table 2.1) at all the temperatures tested.

The separation of the study isolates into two groups by mycelial growth rate was less evident as the temperature of incubation increased. Moreover, mycelial growth rates of the Northern group of isolates (A2 clade I) significantly decreased as temperature increased ($P < 0.01$) with means of 7.4 ± 0.7, 6.8 ± 1, and 4.99 ± 0.1 mm/day at 22℃, 25℃ and 28℃, respectively (Table 2.1). The mean of the growth rates of the Southern isolates (5.1 ± 0.5, 4.9 ± 0.5, and 4.9 ± 0.4 mm/day at 22℃, 25℃and 28℃, respectively) remained the same as incubation temperature increase ($P = 0.143$) suggesting that *P. cinnamomi* isolates collected from Northern California could be more sensitive to higher temperatures when compared with the isolates collected in Southern California. Together, these results suggest variability in mycelial growth rate and optimal growth temperatures among the isolates within and between these two groups of isolates.

Effect of nutrient media on colony growth rate. Mycelial growth rate was determined on two different nutrient media at 22℃ to rule out the possibility that the differences observed within and between groups of isolates were due to the nutrient media composition. There was a significant effect of nutrient media on the mycelial growth of the isolates tested ($P = 0.0142$). However, all the Northern A2 clade I isolates still showed higher mycelial growth rates than the Southern isolates when grown on 10% cV8A or CMA media at 22℃ (Table 2.1), suggesting that the correlation between mycelial growth rate and these two groups of isolates at this incubation temperature was not affected by nutrient media composition.

***In vitro* fungicide sensitivity.** No isolates of *P. cinnamomi* tested in this study were insensitive to potassium phosphite, mefenoxam, fluopicolide, and oxathiapiprolin (Table 2.2). Potassium phosphite had the highest EC_{50} values for mycelial growth inhibition, with ranges and mean values of 19.3 to 34.1 and 24.3 µg/ml for the A2 clade I Northern isolates. On the other hand, higher EC_{50} values were found for all the Southern isolates ranged from 98.9 to > 100 µg/ml, at least more than 4 times than the mean for the Northern isolates. Oxathiapiprolin exhibited the lowest EC_{50} values, with ranges and mean values of 0.0002 to 0.0005 and 0.0003 µg/ml for all the isolates. Mefenoxam and fluopicolide EC_{50} values ranged from 0.05 to 0.10 and 0.07 to 0.17 µg/ml, respectively among the isolates tested. These findings suggest that potassium phosphite sensitivity could be a group characteristic between *P. cinnamomi* isolates collected from Northern California and isolates collected from Southern California. Moreover, in this study we reported for the first time that *P. cinnamomi* avocado isolates from California are

sensitive to fluopicolide and oxathiapiprolin, being the latest the most toxic among all four fungicides tested in this study.

***P. cinnamomi* virulence variability in avocado.** To determine if there are differences in virulence among representative isolates from Northern (N-2110, N-2113, and N-2114) and Southern (S-2109, S-2117, and S-2120) populations from California, two avocado rootstock accessions with contrasting resistance phenotypes were inoculated using the six *P. cinnamomi* isolates individually and in mixtures. Dusa® and PS.54, a moderate resistant and a susceptible rootstock, respectively were used. The percentage (%) of healthy root tissue was significantly different ($\alpha = 0.05$) between the Dusa® and the PS.54 rootstock independent of the treatments. As expected, the root pathogen, *P cinnamomi*, caused more disease in the roots of PS.54, the susceptible avocado rootstock, which exhibited less healthy root tissue (12.58%) after infection compared with Dusa®, the moderate resistant rootstock (46.64%). Disease severities for each treatment as influenced by rootstock and isolate were compared. All isolates and their mixtures caused similar disease severity when a susceptible rootstock (PS.54) was used for inoculation (Fig. 2.1). However, two of the three Northern isolates (N-2110 and N-2113) and the Northern mixture (North Mix) appeared to cause less disease severity compared with the other treatments in this highly susceptible rootstock. On the other hand, when Dusa®, was used for pathogen inoculations, significant differences were detected among treatments (Fig. 2.1). For instance, the S-2109 and S-2117 Southern isolates were significantly more virulent than the N-2110 and N-2113 Northern isolates. The average of the percentage of healthy root tissue for the Southern isolates (27%) was significantly different than the

average of the Northern isolates (57%) (t-test, $P = 0.02$) suggesting that the Southern isolates were more virulent. Furthermore, the Northern and Southern groups of isolates were also differentiated by their virulence when inoculated in Dusa® as mixtures (Fig. 2.1). The South mix (S-2109, S-2117, and S-2120) caused significantly more disease severity than the Northern isolates when inoculated individually or as a mixture (North mix). Interestingly, no significant difference was found between the disease severity caused by the North mix and the mixture of all the six isolates (All Mix) (Fig. 2.1).

Development of *P. cinnamomi* detached leaf inoculation assay using *N. benthamiana*. *P. cinnamomi* successfully infected and colonized *N. benthamiana* leaves exerting an expected hemibiotrophic lifestyle (Fig. 2.2). The infection process of the *P. cinnamomi* isolate N-2113 was followed visually over the course of 120 hours post inoculation (hpi). No visible signs of infection were observed from 3 to 36 hpi (Supplementary Fig. 2.1; Figs. 2.2A to C) and in all the mock inoculated leaves (*data not shown*). Necrotic lesions were observed at 48 hpi and expanded until the last time point assessed (Figs. 2.2D to F). By 120 hpi the whole leaf was complete necrotized (*data not shown*).

At 3 hpi most of the zoospores encysted but did not germinate as revealed by trypan blue staining of the infected tissue. Germinated cysts and subjacent nascent hyphae were visible at 6 and 12 hpi (Fig. 2.2G). Pathogen colonization was observed from 24 to 36 hpi with no microscopic signs of cell death (Figs. 2.2H and I). Pathogen haustoria were visible at 24 and 36 hpi (Figs. 2.2H and I), indicative of its biotrophic growth. Cell death was observed microscopically by trypan blue staining at 48 hpi (Fig.

2.2J), this was in agreement with the development of visible necrotic lesions at the inoculated area by this time point (Fig. 2.2D). Cell death expanded from the inoculated area until the last time point analyzed (Figs. 2.2K and L).

Visible and microscopic observations were confirmed by *P. cinnamomi* DNA quantification in the infected tissue at each time point of inoculation analyzed using TaqMan real-time PCR (Fig. 2.3). *Phytophthora cinnamomi* (N-2113) DNA was used to generate the serial dilution standard curve plot (Fig. 2.3A) that was used to determine the amount of pathogen DNA in the inoculated area at each time point analyzed (Fig. 2.3B). In agreement with the macroscopic and microscopic assessment of the disease progression in *N. benthamiana*, *P. cinnamomi* DNA increased throughout the course of the infection process. Levels began to increase from 6 to 12 hpi and continued to increase until 48 hpi. At 48 hpi the total pathogen DNA no longer increases (Fig. 2.3B). Finally, this *P. cinnamomi* detached leaf inoculation method was validated by detecting significantly differences in virulence among *P. cinnamomi* S-2109 and N-2113 isolates, which exhibited contrasting virulence phenotypes in avocado using the traditional whole plant root inoculation method in Dusa® rootstocks (Fig. 2.1). Similarly, the most virulent isolate (S-2109) formed significant larger necrotic lesions at 3 dpi when compared with the less virulent isolate, N-2113 (Supplementary Fig. 2.2).

DISCUSSION

This is the first study describing the phenotype of *P. cinnamomi* isolates, collected from PRR infected avocado roots, representing the two A2 mating types groups (A2 clade I and II) identified by Paglaccia et al. (2013). The A2 clade I contained *P. cinnamomi* isolates collected from 1989 to 2010, whereas the A2 clade II only contained a unique set of isolates collected in 2009 and 2010 from Southern California avocado growing regions. This study reported significant differences in vegetative growth, fungicide sensitivity, and virulence among all the isolates tested. Moreover, Southern isolates were less sensitive to potassium phosphite and have slower growth rate at 22℃ whereas Northern isolates were more sensitive to potassium phosphite and have higher growth rates. This trend observed suggests a correlation between mycelial growth rate at 22°Cand potassium phosphite sensitivity with specific group of isolates.

The group of Northern isolates corresponding to the A2 clade I mating type group exhibited higher mycelial growth rates than the Southern group of isolates at 22°C independent of the nutrient media used (cV8A or CMA). Consistent with the results of Zentmyer et al. (1976), there was a significant effect of nutrient media and mycelial growth rate of the *P. cinnamomi* study isolates, however this effect did not alter the split of the Northern and Southern isolates by their growth rate. The higher sensitivity of the A2 clade I isolates, collected from Northern California, to higher temperatures when compared with the Southern isolates might be explained by the fact that the median temperature is higher in Southern than in Northern California throughout the year.

Previous studies reported phenotypic differences in colony morphology, growth rate, and optimum growth temperature among *P. cinnamomi* isolates, however the majority of these studies compared isolates from different mating types, origin (countries), and host plants (Eggers et al. 2012; Phillips and Weste 1985; Zentmyer et al. 1976). Only a few studies have included *P. cinnamomi* associated with avocado when assessing phenotypic variability (Chee and Newhook 1965; Lopez- Herrera and Perez-Jimenez 1995; Zentmyer et al. 1976, Zentmyer 1980). In the majority of these studies, the authors could not assign a phenotypic trait to a specific group of isolates. Others have reported varying results on the linkage between genotype groups and colony morphology. Dobrowolski et al. (2003) reported that colony morphology of Australian *P. cinnamomi* isolates grouped with a particularly genotype. In contrast to these results, colony morphology among the twelve isolates tested in this study did not vary significantly. Lopez-Herrera and Perez-Jimenez (1995) reported significant differences on colony morphology among *P. cinnamomi* A2 isolates collected from avocado trees in Spain, but these isolates did not exhibit significant differences on mycelial growth rates. California produces 95% of the avocado crop for the U.S.A and PRR caused by *P. cinnamomi* is responsible for commercial losses totaling $40 million annually statewide. Mefenoxam and phosphite applications are widely used to prevent and combat this disease (Darvas et al. 1984; Duan et al. 2008; Hardy et al. 2001; Hu et al. 2010; Ma and McLeod 2014; Pegg et al. 1985; Shearer and Fairman 2007), however, there is a notably preference for phosphite products over mefenoxam among the growers and this is particularly true for California growers. This preference could explain why mefenoxam-resistant isolates were

not found in this study. Moreover, the range of the EC_{50} values for mefenoxam was consistent with previous values reported for *P. cinnamomi* in the U.S.A (Benson and Grand 2000; Duan et al. 2008; Hu et al. 2010). Duan et al. (2008) reported minor variation in sensitivity to mefenoxam among *P. cinnamomi* isolates collected from diseased ornamental plants in South Carolina. The EC_{50} values of the majority of these isolates were less than 0.1 µg/ml. Hu et al. (2010) found that there were more variations in mefenoxam sensitivity among *P. cinnamomi* isolates collected from different host species than from the same host. In this study, the authors classified isolates as sensitive to mefenoxam when their EC_{50} values ranged from 0.01 to 0.02 µg/ml and as intermediate when EC_{50} values ranged from 0.03 to 0.08 µg/ml. The EC_{50} values for mefenoxam in this study were also less than 0.1 µg/ml suggesting that the repeated use of this fungicide to control PRR in nurseries and avocado orchards does not appear to have reduced the mefenoxam sensitivity of *P. cinnamomi* isolates.

In contrast to mefenoxam, we detected a significant variability among the isolates in potassium phosphite sensitivity but we did not find potassium phosphite-resistant isolates. The Northern isolates corresponding to the A2 clade I group had EC_{50} values of < 34.1 µg/ml, whereas the Southern isolates had EC_{50} values of > 98.9 µg/ml. It is worrisome that the Southern group of isolates including the A2 clade II isolates that exhibited more virulence in the moderate resistant Dusa®, which is the current industry standard rootstock among California avocado growers, were less sensitive to potassium phosphite. Their higher EC_{50} values could represent a selection from higher doses of potassium phosphite being necessary to suppress and control avocado root rot where

these isolates are present. A more detailed study with a larger number of isolates and history of phosphonate applications in the field are needed it to test this hypothesis. Dobrowolski et al. (2008) demonstrated that *P. cinnamomi* isolates exposed to long periods of phosphite treatment in avocado orchards in Australia exhibited reduced sensitivity to phosphite when evaluated on avocado, lupin, and eucalyptus suggesting the onset of resistance to this fungicide. Similar results have been reported for *P. cinnamomi* isolates from avocado orchards in South Africa (Ma and McLeod 2014). This study reports for the first time, the presence of *P. cinnamomi* isolates, collected from PRR diseased avocado roots in California, that are less sensitive to potassium phosphate.

To help delay the development of phosphite-resistant *P cinnamomi* isolates, care should be taken to alternate or mix phosphite products with other effective fungicides with different mode of action to control avocado PRR. Phosphite and mefenoxam rotation with alternative fungicides is commonly used to prevent or reduce the emergence of Phytophthora resistant isolates (Saville et al. 2015), however there are no other fungicides tested or registered to control *P. cinnamomi* in avocado. Fluopicolide and oxathiapiprolin are two new oomycete-targeted fungicides that have been tested for several *Phytophthora* spp., but not for *P. cinnamomi* (Keinath and Kousik 2011; Miao et al. 2016; Patel et al. 2015; Saville et al. 2015). In this study, we report for the first time that the EC_{50} values for mycelial growth inhibition of *P. cinnamomi* avocado isolates are within the range of the EC_{50} values reported for other *Phytophthora* spp. using these two fungicides (Gray et al. 2018; Keinath and Kousik 2011; Miao et al. 2016). Gray et al. (2018) recently reported the Fluopicolide and oxathiapiprolin EC_{50} values for several

Phytophthora species associated with citrus on California. Fluopicolide EC_{50} values for *P. citrophthora*, *P. syringae*, *P. nicotianae*, and *P. hibernalis* ranged from 0.031 to 0.087, 0.02 to 0.0461, 0.039 to 0.095, and 0.017 to 0.018 µg/ml, respectively.

Of the four fungicides evaluated in this study, oxathiapiprolin had the lowest EC_{50} values (0.00016 to 0.00045 µg/ml). This range is similar to EC_{50} values reported for other *Phytophthora* spp. (Gray et al. 2018; Miao et al. 2016) including *P. sojae*, another member of the phylogenetic clade 7, which includes *P. cinnamomi* (Martin et al. 2014). In agreement to our study, Gray et al. (2018), also found that this fungicide had the lowest EC_{50} values among five different fungicides tested. The authors reported oxathiapiprolin EC_{50} values for *P. citrophthora*, *P. syringae*, *P. nicotianae*, and *P. hibernalis* ranged from 0.0002 to 0.0015, 0.0002 to 0.0003, 0.0003 to 0.001, and < 0.0003 µg/ml, respectively.

Interestingly, in contrast to potassium phosphite sensitivity, the Southern isolates in this study exhibited lower EC_{50} values than the Northern A2 clade I isolates. These results suggest the potential to rotate oxathiapiprolin with phosphonates and mefenoxam for controlling avocado PRR in California reducing the risk of the emergence of phosphonate-resistant *P. cinnamomi* isolates.

Significant variation in pathogenicity and virulence among *P. cinnamomi* isolates collected from different host plants have been previously reported (Dudzinski et al. 1993; Linde et al. 1999; Robin and Desprez-Loustau 1998; Zentmyer 1980; Zentmyer and Guillemet 1981). Few cases have reported no differences in virulence among *P. cinnamomi* isolates collected from different hosts (Podger 1989) and unique host

including one study in avocado (Eggers et al. 2012; Zentmyer 1980). In this study we found significant differences in virulence among three representative *P. cinnamomi* isolates from the two A2 clonal groups identified by Paglaccia et al. (2013) when we inoculated a moderate resistant avocado rootstock but not the most susceptible one. The use of highly susceptible plants to distinguish between isolates with different levels of virulence is challenging and could explain why we did not detect significant differences in virulence among our isolates when we used a susceptible avocado rootstock (PS.54). Although the mixture of the A2 clade II isolates (South mix) were more virulent than the mixture of the A2 clade I isolates (North mix), we observed that the mixture of all the isolates together exhibited the same disease severity as the mixture of the less aggressive ones (Fig. 2.1). This observation might be explained by the level of competition between these two groups of might reflect the induction of specific plant defense responses triggered by these two distinct groups of isolates, a more comprehensive study is required to test these two possibilities.

Similar to this study, Linde et al. (1999), reported correlations between *P. cinnamomi* genotypes from Australia and South Africa and the level of virulence in eucalyptus, suggesting that genotype variation may indicate pathogenic variability. On the other hand, Eggers et al. (2012), did not detect differences in pathogenicity or virulence among *P. cinnamomi* isolates from oak forest soils in Eastern United States Zentmyer (1980) did not find significant differences in pathogenicity among twelve A2 California isolates collected from avocado in Northern and Sothern California when inoculated roots of avocado susceptible seedlings.

P. cinnamomi isolates less sensitive to phosphite, and more virulent, exhibited slower mycelial growth rate. Based on these results we speculated that a negative correlation might exist between the *in vitro* growth rates of some *P. cinnamomi* isolates with the level of virulence and sensitivity to fungicides, which could potentially indicate a fitness cost for some traits over other acquired traits. Our results did not support the standard assumption that increased parasite growth leads to increased virulence (classical virulence evolution theory) (Alizon et al. 2009; Anderson and May 1982) but are consistent with an increasing amount of evidence for plant and human pathogens indicating that there is a trade-off between parasite growth and virulence and pathogen survival (Childers et al. 2015; Leggett et al. 2017; Meyer et al. 2010; Thrall et al. 2005). Childers et al. (2015) showed that many isolates of *P. infestans* (hemibiotrophic oomycete) that acquired resistance to mefenoxam exhibited retarded mycelial growth in comparison to the parental isolates that have never been exposed to this fungicide. More research is required to test some of these hypotheses. Similar to this study, Meyer et al. (2010) found that virulence of the necrotrophic fungal pathogen, *Pyrenophora semeniperda,* was significantly negatively correlated with mycelial growth, suggesting a tradeoff between these two traits considering that the production of pathogen toxins necessary to kill the host cells competes with the metabolic processes required for pathogen growth. Similar tradeoff scenarios could be applied for hemibiotrophic pathogens such as *P. cinnamomi* because at the beginning of the interaction with their host plants, hemibiotrophs need to produce effector proteins to avoid plant recognition and suppress the plant immune system at the biotrophic stages. Furthermore, later in the

interaction, these pathogens need to switch to a necrotrophic stage involving the production of another sets of effector proteins to induce host cell death including pathogen toxins (Fawke et al. 2015; Hardham and Blackman 2018).

Variability in pathogenicity and virulence among *P. cinnamomi* isolates has been tested using woody perennial crops such as avocado, eucalyptus, oak, and chestnut (Dudzinski et al. 1993; Linde et al. 1999; Robin and Desprez-Loustau 1998; Zentmyer 1980). There are several limitations in using tree crops to study and characterize pathogenicity and virulence for a large number of *P. cinnamomi* isolates including: (i) the requirement for using genetically uniform clonal material, (ii) only a small number of experiments can be completed annually, (iii) the large greenhouse space required to conduct the experiments, and (iv) the long time (weeks) to score the disease.

Model plants including *Arabidopsis* (Esharghi et al. 2011; Robinson and Cahill 2003), *Lupinus* (Esharghi et al. 2014b; Gunning et al. 2013), and *Medicago* (Huisman et al. 2015) have been previously reported as susceptible hosts for this oomycete pathogen and have been used to study *P. cinnamomi* pathogenesis and plant responses to this pathogen. *N. benthamiana*, is a model plant that has been widely used by the oomycete community to understand *Phytophthora* pathogenicity (Kamoun et al. 1998) and the molecular interaction with their host plants (Goodin et al. 2008). Recently, this model plant has been used to study other hemibiotrophic Phytophthora root pathogens such as *P. palmivora* (Evangelisiti et al. 2017; Khunjan et al. 2016), *P. capsici* (Vega-Arreguín et al. 2014), and the citrus pathogen *P. parasitica* (Dalio et al. 2018). Taking advantage of the extremely wide host range of *P. cinnamomi*, we assessed and found that *P. cinnamomi*

could infect and colonize *N. benthamiana*. While *P. cinnamomi* has been reported on other *Nicotiana* specie in Russia (Erwin and Ribeiro 1996), this study is the first report showing that *N. benthamiana* is also a host for this pathogen. Similar to the detached leaf *P. cinnamomi* inoculation method developed in *Arabidopsis*, we used this detached leaf assay to inoculate *N. benthamiana* leaves with this oomycete. Detached leaf assays offer several advantages over whole plant inoculations including: i) greater reproducibility due to similar size and age of leaves, ii) increased replication, iii) more consisted delivery and localization of the inoculum in the leaves, iv) uniform incubation conditions, v) more accurately quantification of disease, and vi) reduction of space require for inoculations. Robin and Guest (1994) used detached leaf assays to characterize the pathogenicity of *P. parasitica* isolates in four tobacco cultivars. Detached leaf assays have been also used in tree crops to study Phytophthora root pathogens pathogenicity and virulence. Denman et al. (2005) used detached leaf inoculations using different tree species to study the response of several isolates of *P. ramorum.* Helliwel et al. (2016) used detached leaf assays using *Theobroma cacao* to study *P. palmivora*.

In agreement with previous studies using other host plants including avocado, we found that *P cinnamomi* as expected exhibited a hemibiotrophic lifestyle (Fawke et al. 2015; Hardham and Blackman 2018; Huisman et al. 2015; Van den Berg et al. 2018) when inoculated on *N. benthamiana* leaves as supported by our microscopic studies and DNA pathogen quantification using TaqMan real-time PCR. *P. cinnamomi* haustoria-like structure were detected in *N. benthamiana* leaves (Figs. 2.2H and I) as was found in infected roots of other host plants such as avocado (Van den Berg et al. 2018), Medicago

(Huisman et al. 2015); and Quercus (Redondo et al. 2015). Furthermore, the trend of *P. cinnamomi* growth in *N. benthamiana* leaves, determined by DNA quantification using TaqMan quantitative PCR, resembled the pathogen growth in avocado roots monitored using quantitative PCR (Miles et al. 2015). In avocado infected roots, *P. cinnamomi* continuously growth until 7 days post inoculation, then the pathogen growth decreased due to the complete necrosis of the avocado root system. Similarly, *P. cinnamomi* in *N. benthamiana* infected leaves continuously growth until 48 hpi and then decreased due to a complete necrosis of the inoculated area sampled for TaqMan DNA quantification (Figs. 2.2E and F, Fig. 2.3B). Finally, this *N. benthamiana* detached leaf *P. cinnamomi* inoculation method was validated by successfully detecting significant differences in virulence between the S-2109 and the S-2113 isolates, which was consistent with our avocado inoculation results, indicating that this method could be used as an alternative inoculation method to circumvent the difficulties of using whole avocado root inoculation methods to assess the virulence of large number of isolates. All the experiments conducted in this work provide initial evidence of variability in growth rate, optimal growth temperature, fungicide sensitivity, and virulence among isolates representing the two A2 clonal populations identified by Paglaccia et al. (2013). More importantly, the existence of *P. cinnamomi* isolates collected from PRR diseased avocado roots that are more virulent and less sensitive to the current chemical control methods used by avocado growers in California will greatly influence the development of resistant avocado rootstocks and help the implementation of more effective cultural practices for managing

avocado PRR in California including for example the registration of new fungicides for avocado that can be used in combination with mefenoxam and phosphonates.

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REFERENCES

1. Adaskaveg, J. E., Hao, W., and Förster, H. 2015. Postharvest strategies for managing Phytophthora brown rot of citrus using potassium phosphite in combination with heat treatments. Plant Dis. 99:1477-1482.

2. Allardyce, J. A., Rookes, J. E., Hussain, H. I., and Cahill, D. M. 2013. Transcriptional profiling of *Zea mays* roots reveals roles for jasmonic acid and terpenoids in resistance against *Phytophthora cinnamomi*. Funct. Integr. Genomic. 13:217-228.

3. Alizon, S., Hurford, A., Mideo, N., and Van Ballen, M. 2009. Virulence evolution and the trade-off hypothesis: history, current state of affairs, and the future. J. Evol. Biol. 22:245-259.

4. Anderson, R. M. and May, R. M. 1982. Coevolution of hosts and parasites. Parasitology 85:411-426.

5. Andreassi J. L., II., Gutteridge S., Pember, S. O., and Sweigard, J. A. 2013. Detection and screening method and materials useful in performance thereof. International Patent No. WO2013009971. World Intellectual Property Organization.

6. Beaulieu, J., Ford, B., and Balci, Y. 2017. Genotypic Diversity of *Phytophthora cinnamomi* and *P. plurivora* in Maryland Maryland's nurseries and Mid-Atlantic forests. Phytopathology 107:769-776.

7. Benson, D. M., and Grand, L. F. 2000. Incidence of Phytophthora root rot of Fraser fir in North Carolina and sensitivity of isolates of *Phytophthora cinnamomi* to metalaxyl. Plant Dis. 84:661-664.

8. Bilodeau, G. J., Martin, F. N., Coffey, M. D., and Blomquist, C. L. 2014. Development of a multiplex assay for genus- and species-specific detection of *Phytophthora* based on differences in mitochondrial gene order. Phytopathology 104:733-748.

9. Boesewinkel, H. J. 1976. Storage of fungal cultures in water. Trans. Br. Mycol. Soc. 66:183-185.

10. Bombarely, A., Rosli, H., Verbalov, J., Moffett, P., Mueller, L. A., and Martin, G. B. 2012. A draft genome sequence of *Nicotiana benthamiana* to enhance

molecular plant-microbe biology research. Mol. Plant Microbe Interact. 25:1523-1530.

11. Briggs, G., Mansfield, D., Moloney, B., Gary, S., and Wegmann, T. 2006. The discovery and chemistry of fluopicolide: A new standard for oomycetes disease control. Pflanzenschutz-Nachr. Bayer 59:141-152.

12. Brunner, I., Brodbeck, S., Büchler, U., and Sperisen, C. 2001. Molecular identification of fine roots of trees from the Alps: reliable and fast DNA extraction and PCR–RFLP analyses of plastid DNA. Mol. Ecol. 10:2079-2087.

13. Chee, K. and Newhook, F. 1965. Variability in *Phytophthora cinnamomi* Rands. New Zeal. J. Agr. Res. 8:96-103.

14. Chen, D., and Zentmyer, G. A. 1970. Production of sporangia by *Phytophthora cinnamomi* in axenic culture. Mycologia 62:397-401.

15. Chen, X., Xing, Y., Li, Y., Tomg, Y., and Xu, J. 2013. RNA-Seq reveals infection-related gene expression changes in *Phytophthora capsici*. PLOS one 8(9):e74588.

16. Childers, R., Danies, G., Myers, K., Fei, Z., Small, I. M, and Fry W. E. 2015. Acquired resistance to mefenoxam in sensitive isolates of *Phytophthora infestans*. Phytopathology 105:342-349.

17. Coffey, M. D., and Bower, L. A. 1984. *In vitro* variability among isolates of eight *Phytophthora* species in response to phosphorous acid. Phytopathology 74:738-742.

18. Dalio, R. J., Maximo, H. J., Oliveira, T. S., Dias, R. O., Breton, M. C., Felizatti, H., and Machado, M. 2018. *Phytophthora parasitica* effector PpRxLR2 suppresses *Nicotiana benthamiana* immunity. MPMI 31:481-493.

19. Darvas, J. M., Toerien, J. C., and Mine, D. L. 1984. Control of avocado root rot by trunk injection with phosethyl-Al. Plant Dis. 68:691-693.

20. Denman, S., Kirk, S. A., Brasier, C. M., and Webber, J. F. 2005. *In vitro* leaf inoculations studies as an indication of tree foliage susceptibility to *Phytophthora ramorum* in the UK. Plant Pathol. 54:512-521.

21. Dobrowolski, M., Tommerup, I. C., Shearer, B. L., and O'Brien, P. A. 2003. Three clonal lineage of Phytophthora cinnamomi in Australia revealed by microsatellites. Phytopathology 93:695-704.

22. Dobrowolski, M. P., Shearer, B. L., Colquhoun, I. J., O'Brien, P. A., and Hardy, G. E. 2008. Selection for decreased sensitivity to phosphite in *Phytophthora cinnamomi* with prolonged use of fungicide. Plant Pathol. 57:928-936.

23. Dong, L., Cheng, Y., Wu, J., Cheng, Q., Li, W., Fan, S., Jiang, L., Xu, Z., Kong, F., Zhang, D., Xu, P., and Zhang, S. 2015. Overexpression of GmERF5, a new member of the soybean EAR motif-containing ERF transcription factor, enhances resistance to *Phytophthora sojae* in soybean. J. Exp. Bot. 66:1-13.

24. Drenth, A., and Sendall, B. 2001. Inoculation of soil. Pages 37-38 in: Practical guide to detection and identification of *Phytophthora*. CRC for Tropical Plant Protection, Brisbane, Australia.

25. Duan, C. H., Riley, M. B., and Jeffers, S. N. 2008. Characterization of *Phytophthora cinnamomi* populations from ornamental plants in South Carolina, USA. Arch. Phytopathol. Plant Protect. 41:14-30.

26. Dudzinski, M. J., Old, K. M.; and Gibbs, R. J. 1993. Pathogenic variability in Australian isolates of *Phytophthora cinnamomi.* Australas. J. Bot. 41:721-732.

27. Eckert, J. W., and Tsao, P. H. 1962. A selective medium for isolation of *Phytophthora* and *Pythium* from plant roots, Phytopathology 52:771.

28. Eggers, J. E., Balci, Y., and MacDonald, W. L. 2012. Variation among *Phytophthora cinnamomi* isolates from oak forest soils in the eastern United States. Plant Dis. 96:1608-1614.

29. Ekchaweng, K., Evangelisti, E., Schornack, S., Tian, M., and Churngchow. 2017. The plant defense and pathogen counterdefense mediated by *Hevea brasilensis* serine protease *HbSPA* and *Phytophthora palmivora* extracellular protease inhibitor *PpEPI10*. PLOS one 12(5):e0175795.

30. Engelbrecht, J., Duong, T. A., and Van der Berg, N. 2017. New microsatellite markers for population studies of *Phytophthora cinnamomi*, an important global pathogen. Sci. Rep. 7:17631.

31. Erwin, D. C., and Ribeiro, O. K. 1996. Phytophthora Diseases Worldwide. APS Press, The American Phytopathological Society, St. Paul, MN.

32. Esharghi, L., Anderson, J., Aryamanesh, N., Shearer, B., McComb, J., Hardy, G. E. St. J., and O'Brien, P. A. 2011. Phosphite primed defense responses and enhanced expression of defense genes in *Arabidopsis thaliana* infected with *Phytophthora cinnamomi*. Plant Pathol. 60:1086-1095.

33. Eshraghi, L., Anderson, J. P., Aryamanesh, N., Mccomb, J. A., Shearer, B. L., and Hardy, G. E. St. J. 2014a. Defence signalling pathways involved in plant resistance and phosphite-mediated control of *Phytophthora cinnamomi*. Plant Mol. Biol. Rep. 32:342-356.

34. Eshraghi, L., Anderson, J. P., Aryamanesh, N., McComb, J. A., Shearer, B., and Hardy, G. E. St. J. 2014b. Suppression of the auxin response pathway enhances susceptibility to *Phytophthora cinnamomi* while phosphite-mediated resistance stimulates the auxin signalling pathway. BMC Plant Biol. 14:68.

35. Evangelisti, E., Gogleva, A., Hainaux, T., Doumane, M., Tulin, F., Quan, C., and Schornack, S. 2017. Time-resolved dual transcriptomics reveal early induced *Nicotiana benthamiana* root genes and conserved infection-promoting *Phytophthora palmivora* effectors. BMC Biol. 15:39.

36. Fawke, S., Doumane, M., and Schornack, S. 2015. Oomycete interactions with plants: infection strategies and resistance principles. Microbiol. Mol. Biol. Rev. 79:263-280.

37. Fenn, M. E., and Coffey, M. D. 1984.Studies on the *in vitro* and in vivo antifungal activity of fosetyl-A1and phosphorous acid. Phytopathology 74:606-611.

38. Förster, H., Kanetis, L., and Adaskaveg, J. E. 2004. Spiral gradient dilution, a rapid method for determining growth responses and 50% effective concentration values in fungus-fungicide interactions. Phytopathology 94:163-170.

39. Goodin, M. M., Zaitlin, D., Naidu, R. A., and Lommel, S. A. 2008. *Nicotiana benthamiana*: its history and future as a model for plant-pathogen interactions. MPMI 21:1015-1026.

40. Gray, M., Hao, W., Förster, H., and Adaskaveg, J. E. 2018. Baseline sensitivities of new fungicides and their toxicity to selected life stages of *Phytophthora* species from citrus in California. Plant Dis. 102:734-742.

41. Guest, D. and Grant, B. R. 1991. The complex action of phosphonates as antifungal agents. Biol. Rev. 66:159-187.

42. Gunning, T. K., Conlan, X. A., Parker, R. M., Dyson, G. A., Adams, M. J., Barnett, N. W., and Cahill, D. M. 2013. Profiling of secondary metabolites in blue lupin inoculated with *Phytophthora cinnamomi* following phosphite treatment. Funct. Plant Biol. 40:1089-1097.

43. Hardham, A. R. 2005. *Phytophthora cinnamomi*. Mol. Plant Pathol. 6:589-604.

44. Hardham, A. and Blackman, L. 2018. *Phytophthora cinnamomi*. Mol. Plant Pathol. 19:260-285.

45. Hardy, G. E. St. J., Barrett, S., and Shearer, B. L. 2001. The future of phosphite as a fungicide to control the soilborne plant pathogen *Phytophthora cinnamomi* in natural ecosystems. Australas. Plant Pathol. 30:133-139.

46. Helliwell, E. E., Vega-Arreguin, J., Shi, Z., Bailey, B., Xiao, S., Maximova, S. N., Tyler, B. M., and Guiltinan, M. J. 2016. Enhanced resistance in *Theobroma cacao* against oomycete and fungal pathogens by secretion of phosphatidylinositol-3-phosphate-binding proteins. Plant Biotechnol. J. 14:875-886.

47. Hu, J., Hong, C., Stromberg, E. L., and Moorman, G. W. 2010. Mefenoxam sensitivity in *Phytophthora cinnamomi* isolates. Plant Dis. 94:39-44.

48. Huisman, R., Bouwmeester, K., Brattinga, M., Govers, F., Bisseling, T., and Limpens, E. 2015. Haustorium formation in *Medicago truncatula* roots infected by *Phytophthora palmivora* does not involve the common endosymbiotic program shared by arbuscular mycorrhizal fungi and rhizobia. MPMI 28:1271-1280.

49. Kamoun, S., van West, P., Vleeshouwers, V., de Groot, K. E., and Govers, F. 1998. Resistance of *Nicotiana benthamiana* to *Phytophthora infestans* is mediated by the recognition of the elicitor protein INF1. Plant Cell 10:1413-1426.

50. Kamoun, S., Furzer, O., Jones, J. D. G., Judelson, H. S., Ali, G. S., Dalio, R. J., Roy, S. G., Schena, L., Zambounis, A., Panabières, F., Cahill, D., Ruocco, M., Figueiredo, A., Chen, X. R., Hulvey, J., Stam, R., Lamour, K., Gijzen, M., Tyler, B. M., Grünwald, N. J., Mukhtar, M. S., Tomé, D. F., Tör, M., Van Den Ackerveken, G. , McDowell J., Daayf, F., Fry, W. E., Lindqvist-Kreuze. H., Meijer, H. J., Petre, B., Ristaino, J., Yoshida, K., Birch, P. R., and Govers, F. 2015. The Top 10 oomycete pathogens in molecular plant pathology. Mol. Plant Pathol. 16:413-434.

51. Keinath, A. P., and Kousik, C. S. 2011. Sensitivity of isolates of *Phytophthora capsici* from the eastern United States to fluopicolide. Plant Dis. 95:1414-1419.

52. Kroon, L., Bakker, F., van den Bosch, G., Bonants, P., and Flier, W. 2004. Phylogenetic analysis of *Phytophthora* species based on mitochondrial and nuclear DNA sequences. Fungal Genet. Biol. 41:766-782.

53. Khunjan, U., Ekchaweng, K., Panrat, T., Tian, M., and Churngchow, N. 2016. Molecular cloning of HbPR-1 gene from rubber tree, expression of HbPR-1 gene in *Nicotiana benthamiana* and its inhibition of *Phytophthora palmivora*. PloS ONE 11(6):e0157591.

54. Lamour, K. H., and Hausbeck, M. K. 2001. The dynamics of mefenoxam insensitivity in a recombining population of *Phytophthora capsici* characterized with amplified fragment length polymorphism markers. Phytopathology 91:553-557.

55. Leggett, H. C., Cornwallis, C. K., Buckling, A., and West, S. A. 2017. Growth rate, transmission mode and virulence in human pathogens. Phil. Trans. R. Soc. B. Biol. Sci. 372(1719):20160094.

56. Linde, C., Drenth, A., Kemp, G. H., Wingfield, M. J., and von Bromembsen, S. L. 1997. Population structure of *Phytophthora cinnamomi* in South Africa. Phytopathology 87:822-827.

57. Linde, C., Kemp, G. H. J., and Wingfield, M. J. 1999. Variation in pathogenicity among South African isolates of *Phytophthora cinnamomi*. Eur. J. Plant Pathol. 105:231-239.

58. Londsdale, J. H., Botha, T., and Kotze, J. M. 1988. Preliminary trials to assess the resistance of three clonal avocado rootstocks to crown canker caused by *Phytophthora cinnamomi*. South African Avocado Growers' Association Yearbook 11:35-37.

59. Ma, J., and McLeod, A. 2014. *In vitro* sensitivity of South African *Phytophthora cinnamomi* to phosphite at different phosphate concentrations. South African Avocado Growers' Association Yearbook 37:79-84.

60. Manosalva, P., Manohar, M., Von Reuss, S. H., Chen, S., Koch, A., Kaplan, F., Choe, A., Micikas, R. J., Wang, X., Kogel, K. H., Sternberg, P. W., Williamson, V. M., Schroeder, F. C., and Klessig, D. F. 2015. Conserved nematode signaling molecules elicit plant defenses and pathogen resistance. Nat. Commun. 6:7705.

61. Martin, F. N., and Coffey, M. D. 2012. Mitochondrial haplotype analysis for differentiation of isolates of *Phytophthora cinnamomi*. Phytopathology 102:229-239.

62. Martin, F. N, Blair, J. E., and Coffey, M. D. 2014. A combined mitochondrial and nuclear multilocus phylogeny of the genus *Phytophthora.* Fungal Genet. Biol. 66:19-32.

63. McGrath, M. T. 2004. What are fungicides? The plant health instructor. The American Phytopathological Society. doi: 10.1094/PHI-I-2004-0825-01. Online publication. https://www.apsnet.org/edcenter/intropp/topics/Pages/Fungicides.aspx.

64. Mendiburu, F. de. 2015. agricolae: Statistical Procedures for Agricultural Research. R Package Version 1.2-3. http://CRAN.R-project.org/package=agricolae.

65. Meyer, S. E., Stewart, T. E., and Clement, S. 2010. The quick and the deadly: growth vs. virulence in a seed bank pathogen. New Phytol. 187:209-216.

66. Miao, J., Dong, X., Lin, D., Wang, Q., Liu, P., Chen, F., Du, Y. and Liu, X. 2016. Activity of the novel fungicide oxathiapiprolin against plant-pathogenic oomycetes. Pest. Manag. Sci. 72:1572-1577.

67. Miles, T. D., Martin, F. N., and Coffey, M. D. 2015. Development of rapid isothermal amplification assays for detection of *Phytophthora* spp. in plant tissue. Phytopathology 105:265-278.

68. Miles, T. D., Martin, F. N., Robideau, G., Bilodeau, G., and Coffey, M. D. 2017. Systematic development of *Phytophthora* species-specific mitochondrial diagnostic markers for economically important members of the genus. Plant Dis. 101:1162-1170.

69. Ouimette, D. G., and Coffey, M. D. 1989. Comparative antifungal activity of four phosphonate compounds against isolates of nine *Phytophthora* species. Phytopathology 79:761-767.

70. Pagliaccia, D., Pond, E., McKee, B., and Douhan, G. W. 2013. Population genetic structure of *Phytophthora cinnamomi* associated with avocado in California and the discovery of a potentially recent introduction of a new clonal lineage. Phytopathology 103:91-97.

71. Pasteris R. J., Hanagan, M. A., Bisaha, J. J., Finkelstein, B. L., Hoffman, L. E., Gregory, V., Andreassi, J. L., Sweigard, J. A., Klyashchitsky, B. A., Henry, Y. T., and Berger, R. A. 2016. Discovery of oxathiapiprolin, a new oomycete fungicide that targets an oxysterol binding protein. Bioorg. Med. Chem. 24: 354-361.

72. Patel, J. S., Costa de Novaes, M. I., and Zhang, S. 2015. Evaluation of the new compound oxathiapiprolin for control of downy mildew in basil. Plant Health Prog. 16:165-172.

73. Pegg, K. G., Whiley, A. W., Saranah, J. B., and Glass, R. J. 1985. Control of Phytophthora root rot of avocado with phosphorus acid. Australas. Plant Pathol. 14:25-29.

74. Phillips, D, and Weste, G. M. 1985. Growth rates of four Australian isolates of *Phytophthora cinnamomi* in relation to temperature. Trans. Br. Myc. Soc. 84:183-185.

75. Pliego-Alfaro, F. and Litz, R. E. 2007. Biotechnology and avocado improvement. California Avocado Society Yearbook. 90:149-167.

76. Ploetz, R. C. 2013. Phytophthora root rot of avocado. Pages 197-203 in: *Phytophthora*: A Global Perspective. K. Lamour, ed. CABI Plant Protection Series, Vol. 2. CABI, Boston, MA.

77. Podger, F. D. 1989. Comparative pathogenicity of fourteen Australian isolates of *Phytophthora cinnamomi* determined on transplants of Tasmanian temperate heathland. Aust. J. Bot. 37:491-500.

78. R Core Team. 2015. R: A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing. http://www.R-project.org.

79. Redondo, M., Perez-Sierra, A., Abad-Campos, P., Torres, L., Solla, A., Reig-Armiñana, and Garcia-Breijo, F. 2015. Histology of *Quercus ilex* roots during infection by *Phytophthora cinnamomi*. Trees 29:1943-1957.

80. Reitmann, A., Berger, D. K., and Van den Berg, N. 2017. Putative pathogenicity genes of *Phytophthora cinnamomi* identified via RNA-Seq analysis of pre-infection structures. Eur. J. Plant Pathol. 147:211-228.

81. Robin, C., Desprez-Loustau, M. L., Capron, G., and Delatour, C. 1998. First record of *Phytophthora cinnamomi* on cork and holm oaks in France and evidence of pathogenicity. Ann. Sci. For. 55:869-883.

82. Robin, C. and Guest, D. 1994. Characterization of pathogenicity of *Phytophthora parasitica* isolates by stem and detached-leaf inoculations in four tobacco cultivars. New Zeal. J. Crop Hort. 22:159-166.

83. Robinson, L. H., and Cahill, D. M. 2003. Ecotypic variation in the response of *Arabidopsis thaliana* to *Phytophthora cinnamomi*. Australas. Plant Pathol. 32:53-64.

84. Rookes J. E., Wright, M. L., and Cahill, D. M. 2008. Elucidation of defense responses and signaling pathways induced in *Arabidopsis thaliana* following challenge with *Phytophthora cinnamomi*. Physiol. Mol. Plant Pathol. 72:151-161.

85. Saville, A., Graham, K., Grünwald, N. J., Myers, K., Fry, W. E., and Ristaino, J. B. 2015. Fungicide sensitivity of US genotypes of *Phytophthora infestans* to six oomycete-targeted compounds. Plant Dis. 99:659-666.

86. Schwinn, F. J. and Staub, T. 1995. Oomycetes fungicides: phenylamides and other fungicides against Oomycetes. Pages 323-346 in: Modern Selective Fungicides, 2nd ed. H. Lyr ed. Longman Scientific and Technical, Harlow, UK.

87. Shands, A. C., Yamagata, J. S., Wright, A. F., and Miles, T. D. 2016. First report of *Phytophthora cinnamomi* causing root rot of Southern highbush blueberry in California. Plant Dis. 100:537.

88. Shearer, B. L., and Fairman, R. G. 2007. A stem injection of phosphite protects *Banksia* species and *Eucalyptus marginata* from *Phytophthora cinnamomi* for at least four years. Australas. Plant Pathol. 36:78-86.

89. Shew, H. D. 1985. Response of *Phytophthora parasitica* var. *nicotianae* to metalaxyl exposure. Plant Dis. 69:559-562.

90. *Staub*, T. H., and *Young*, T. R. *1980*. Fungitoxicity of metalaxyl against *Phytophthora parasitica* var. *nicotianae*. Phytopathology 70:797-801.

91. Thrall, P. H., Barrett, L. G., Burdon, J. J. and Alexander, H. M. 2005. Variation in pathogen aggressiveness within a metapopulation of the *Cakile maritima–Alternaria brassicicola* host–pathogen association. Plant Pathol. 54:265-274.

92. Van den Berg, N., Christie, J. B., Aveling, T. A. S., and Engelbrecht, J. 2018. Callose and β-1-3 glucanase inhibit *Phytophthora cinnamomi* in a resistant avocado rootstock. Plant Pathol. 67:1150-1160.

93. Vega-Arreguín, J. C., Jalloh, A., Bos, J. I., and Moffett, P. 2014. Recognition of an Avr3a homologue plays a major role in mediating nonhost resistance to *Phytophthora capsici* in *Nicotiana* species. MPMI 27:770-780.

94. Zentmyer, G. A. 1980. *Phytophthora cinnamomi* and the diseases it causes. Monograph No. 10. The American Phytopathological Society, St. Paul, MN.

95. Zentmyer, G. A. 1981. The effect of temperature on growth and pathogenesis of *Phytophthora cinnamomi* and on growth of its avocado host. Phytopathology 71:925-928.

96. Zentmyer, G. A., and Mircetich, S. M. 1966. Saprophytism and persistence in soil by *Phytophthora cinnamomi*. Phytopathology 56:710-712.

97. Zentmyer, G. A., Leary, J. V., Klure, L. J., and Grantham, G. L. 1976. Variability in growth in *Phytophthora cinnamomi* in relation to temperature. Phytopathology 66:982-986.

98. Zentmyer, G. A., and Guillemet, F. B. 1981. Evidence for strains of *Phytophthora cinnamomi*. Plant Dis. 65:475-477.

Table 2.1. Effect of temperatures and nutrient media on mycelial growth of *Phytophthora cinnamomi* isolates on 10% clarified V8 agar (cV8A) and corn meal agar (CMA)

P. cinnamomi		**Mycelial radial growth (mm/day)**			
isolate	**Clade**	**22°C (CMA)**	**22°C (cV8A)**	**25°C (cV8A)**	**28°C (cV8A)**
S-2109	II	5.13±0.07[xy] ef	4.94±0.04 g	4.56±0.06 fg	4.66±0.03 c
S-2117	II	5.16±0.40 ef	5.59±0.27 f	5.28±0.38 e	5.09±0.26 b
S-2120	II	5.38±0.09 e	5.88±0.05 f	5.66±0.11 e	5.50±0.11 a
S-2118	II	4.13±0.14 g	4.47±0.12 h	4.34±0.03 g	4.72±0.06 c
S-st5b	Unknown[z]	4.81±0.15 f	5.06±0.11 g	4.84±0.06 f	4.63±0.05 c
S-st6b	Unknown[z]	4.75±0.05 f	4.63±0.05 gh	4.53±0.12 fg	4.66±0.09 c
N-2110	I	5.97±0.08 d	6.72±0.11 de	5.63±0.09 e	4.72±0.14 c
N-2113	I	8.22±0.14 a	7.66±0.38 b	7.66±0.09 b	5.16±0.03 b
N-2114	I	7.97±0.14 ab	8.44±0.08 a	8.28±0.09 a	5.09±0.18 b
N-B9	I	7.63±0.14 bc	7.16±0.03 cd	7.13±0.09 c	5.13±0.05 b
N-Vor10	I	6.06±0.08 d	6.66±0.06 e	5.69±0.11 e	4.91±0.13 bc
N-Van3	I	7.34±0.16 c	7.59±0.14 bc	6.66±0.17 d	4.94±0.04 bc

[x] Mean values ± stander error.

[y] Numbers followed by the same letter do not differ significantly according to Fisher's Least Significance Difference test at $P = 0.05$.

[z] Isolates were collected from the same field as isolates 2109 and 2117 in 2010, but these isolates were not genotyped or included in Paglaccia et al. 2013.

Table 2.2. Fungicide effective concentrations at which 50% of the mycelial growth (EC_{50} values) of the *Phytophthora cinnamomi* isolates tested in this study

	Fungicide EC_{50} (µg/ml)			
***P. cinnamomi* isolate**	**Potassium phosphite[y]**	**Mefenoxam[z]**	**Oxathiapiprolin[z]**	**Fluopicolide[z]**
S-2109	> 100	0.05502 b	0.00028 cd	0.12521 ab
S-2117	> 100	0.06127 ab	0.00018 ef	0.10641 bc
S-2120	> 100	0.06811 ab	0.00016 f	0.09923 bc
S-2118	98.93 a	0.07086 ab	0.00029 bcd	0.09685 bc
S-st5b	> 100	0.05003 b	0.00022 def	0.08901 bc
S-st6b	> 100	0.05002 b	0.00024 def	0.10239 bc
N-2113	19.62 e	0.07148 ab	0.00044 a	0.13675 ab
N-2110	34.06 b	0.09463 a	0.00038 ab	0.11455 bc
N-2114	24.24 d	0.09540 a	0.00026 cde	0.16683 a
N-B9	19.25 e	0.07215 ab	0.00031 bcd	0.09090 bc
N-Vor10	28.53 c	0.04719 b	0.00034 bc	0.07263 c
N-Van3	19.97 c	0.04283 b	0.00045 a	0.13301 ab

[y] Agar dilution method was used to determine EC_{50} values for inhibition of mycelial growth for potassium phosphite in 10% cV8A. Isolates with a calculated EC_{50} greater than 100 µg/ml (highest concentration tested) are designated as > 100.

[z] Spiral gradient dilution method was used to determine EC_{50} values for inhibition of mycelial growth for each fungicide tested in 10% cV8A.

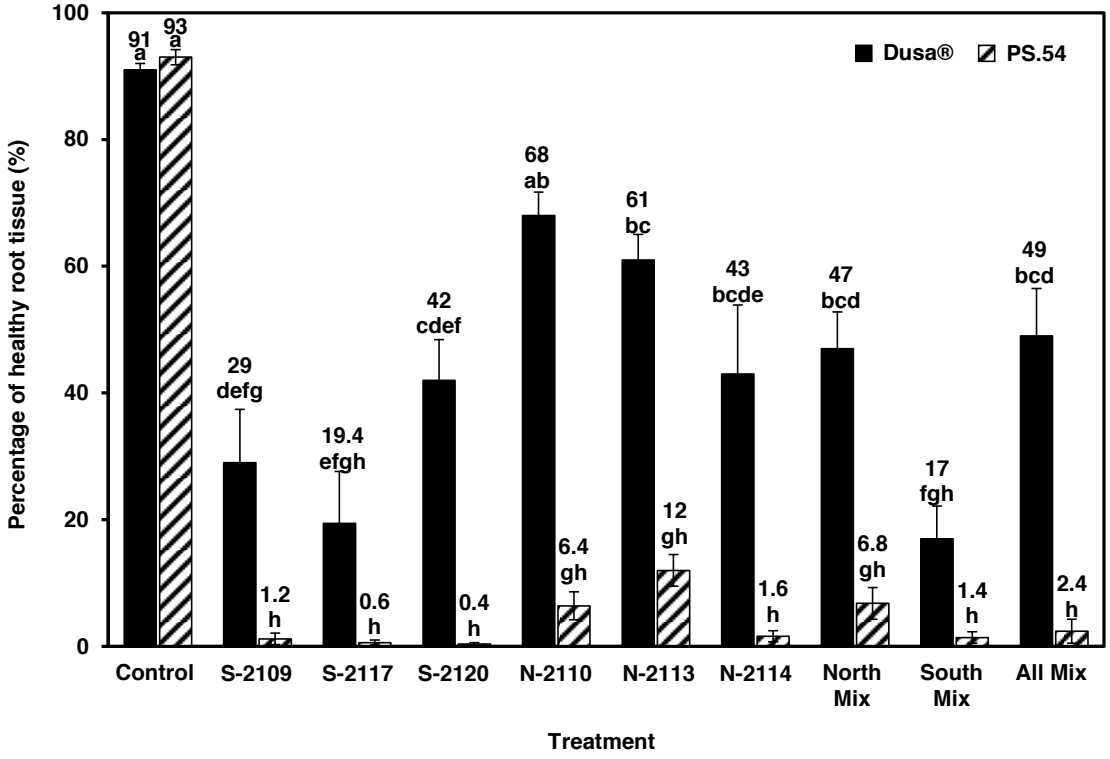


Figure 2.1. Virulence test of *Phytophthora cinnamomi* isolates individually and in mixtures on moderate resistance (Dusa®) and susceptible (PS.54) avocado rootstocks. Disease ratings were measured as percentage of healthy root tissue 17 weeks after inoculation. Disease severity was measured as percentage of healthy root tissue after pathogen root inoculation. More virulent isolates caused higher disease severity (less percentage of healthy root tissue). Error bars indicate standard errors of the means of five replicates. Means are indicated as numbers. Means with the same letter were not significantly different according to the Tukey's HSD test. The experiment was repeated at least three times, with similar results.

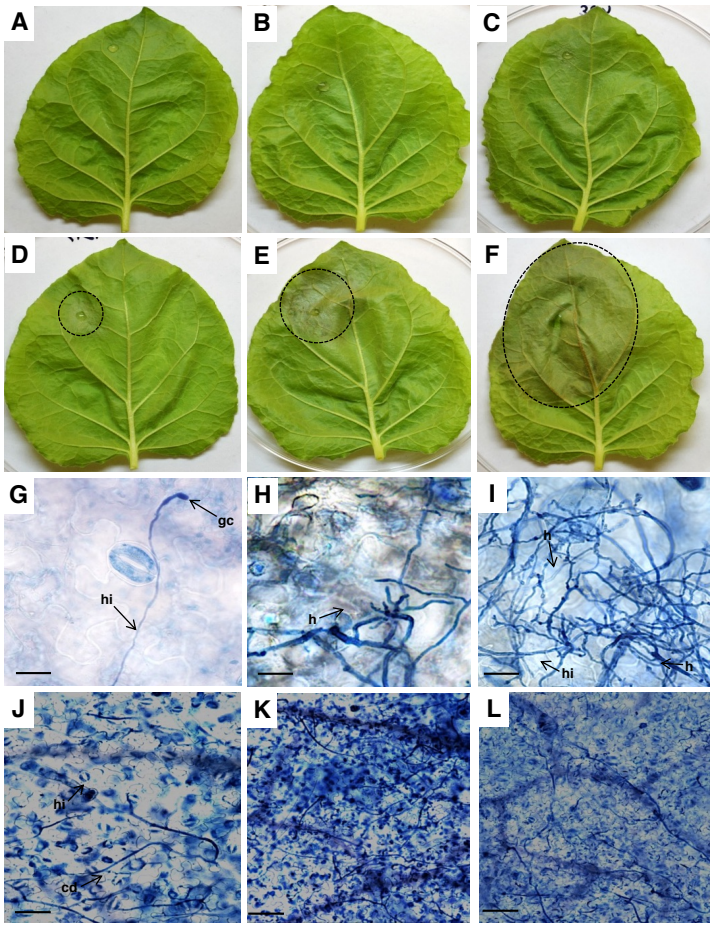


Figure 2.2. Disease progression in *Nicotiana benthamiana* leaves inoculated with the N-2113 isolate of *Phytophthora cinnamomi* using a detached leaflet assay. **A** to **F,** photographs were taken at 12, 24, 36, 48, 72, and 96 hours post inoculation (hpi), respectively. Necrotic lesions are circled. Scale bar = 80 µm. **G** to **L,** light microscopy sections of leaves stained with trypan blue at 12, 24, 36, 48, 72, and 96 hpi, respectively. Scale bar = 20 µm. gc = germinated cyst, hi = hyphae, h = haustoria, cd = cell death.

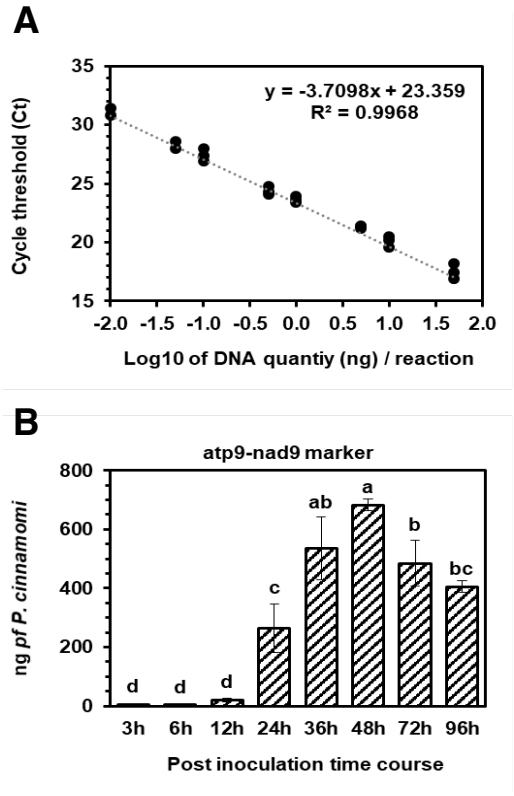


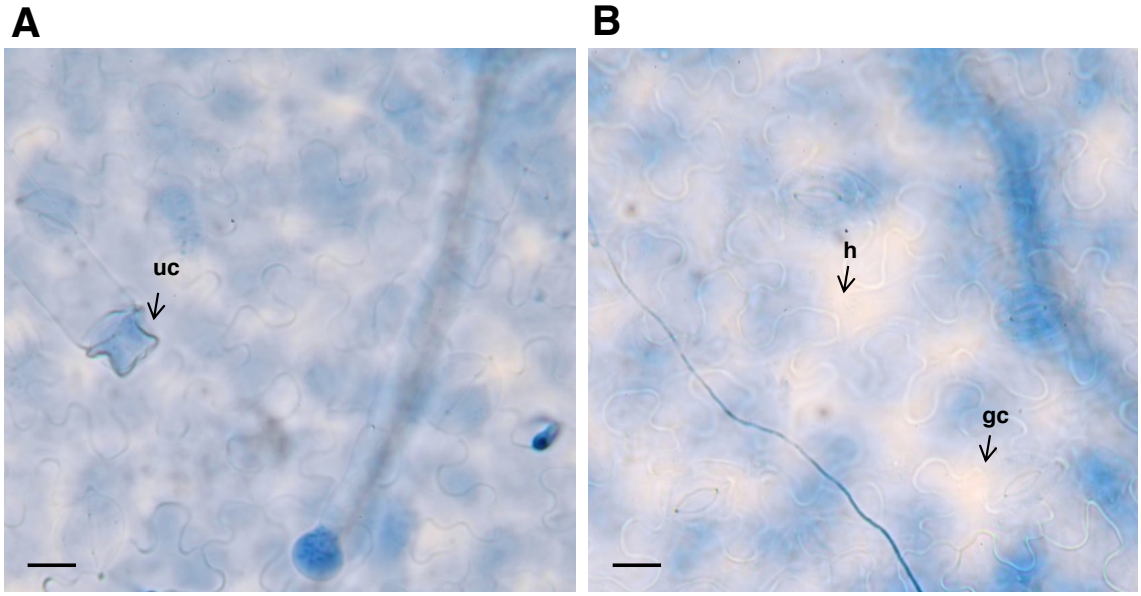
Figure 2.3. *Phytophthora cinnamomi* DNA quantification *in Nicotiana benthamiana* leaves inoculated with N-2113 zoospores. **A,** Serial dilution standard curve plot generated using purified genomic DNA of isolate N-2113 of *P. cinnamomi* using the mitochondrial *atp9-nad9* marker. Error bars indicate standard errors of the means of three technical replications. **B,** Quantification in nanograms (ng) using the mitochondrial *atp9-nad9* marker. Error bars indicate standard errors of the means of three biological replicates and two technical replications. Pathogen detection at a concentration of 4.67, 3.95, and 19.63 ng for 3, 6, 12 hours post inoculation (hpi), respectively. Columns with the same letter indicate that isolate means were not significantly different ($P < 0.05$) following an analysis of variance and Fisher's Least Significance Difference means separation procedures.

Supplementary Table 2.1. Isolates of *Phytophthora cinnamomi* used in this study

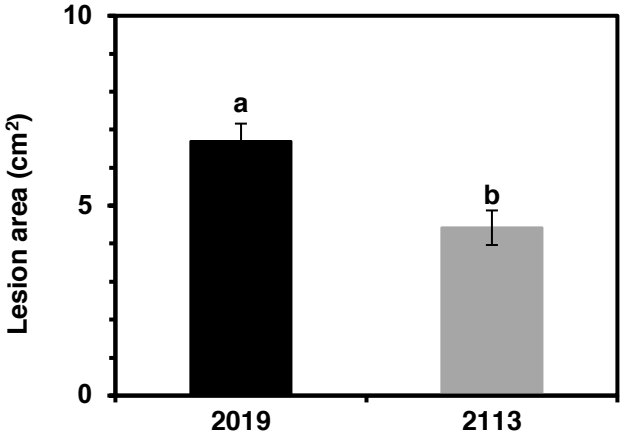
Isolate	**Year collected**	**City of origin**	**Clade**	**Genotype**
S-2109	2009	Pauma Valley	II	S5[y]
S-2117	2009	Pauma Valley	II	S6[y]
S-2120	2009	Temecula	II	S3[y]
S-2118	2010	Pauma Valley	II	S6[y]
S-st5b	2010	Pauma Valley	Unknown	Unknown[x]
S-st6b	2010	Pauma Valley	Unknown	Unknown[x]
N-2110	2010	Ventura	I	N3[y]
N-2113	2010	Ventura	I	N7[y]
N-2114	2009	Ventura	I	N4[y]
N-B9	2010	Santa Barbara	I	N6[y]
N-Vor10	2010	Ventura	I	N3[y]
N-Van3	2010	Ventura	I	N2[y]

[y] Paglaccia et al. 2013.

[x] Isolates were collected from the same ranch as isolates 2109 and 2117 in 2010, but these isolates were not genotyped or included in Paglaccia et al. 2013.



Supplementary Figure 2.1. Light microscopy images of *Nicotiana benthamiana* leaves inoculated with *Phytophthora cinnamomi* zoospores and stained with trypan blue. **A,** 3 hours post inoculation (hpi) and **B,** at 6 hpi. Un-germinated cysts are visible at 3 hpi (uc) and germinated cysts (gc) with hyphae (h) are visible at 6 hpi. Scale bar = 80 µm.



Supplementary Figure 2.2. Lesion size produced by *Phytophthora cinnamomi* isolates in *Nicotiana benthamiana*. Leaves from five-week-old *N. benthamiana* were inoculated with a zoospore suspension (1×10^4 zoospores/ml) of isolates S-2109 and N-2113. Lesion size was measured at 3 days post inoculation (dpi). Error bars indicate standard errors of the means of three replicates. Means with the same letter were not significantly different according to the analysis of variance and Fisher's Least Significance Difference test ($P = 0.05$). The experiment was repeated at least two times, with similar results.

CHAPTER III. NEW OOMYCOTA FUNGICIDES WITH ACTIVITY AGAINST PHYTOPHTHORA CINNAMOMI AND THEIR POTENTIAL USE FOR MANAGING AVOCADO ROOT ROT IN CALIFORNIA

Belisle, R. J., Hao, W., McKee, B., Arpaia, M. L., Manosalva, P., and Adaskaveg, J. E. 2018. New Oomycota fungicides with activity against *Phytophthora cinnamomi* and their potential use for managing avocado root rot in California. Plant Disease (*in review*).

ABSTRACT

Phytophthora root rot (PRR), caused by *Phytophthora cinnamomi*, is the most destructive disease of avocado worldwide. In the United States, mefenoxam and phosphonate products are currently the only registered fungicides for managing avocado PRR. Four new Oomycota-specific and the two registered fungicides, all with different modes of action, were evaluated. Seventy-one isolates of *P. cinnamomi* from avocado in California, most of them recently collected, were tested for their *in vitro* sensitivity to the six fungicides. Baseline sensitivity ranges and mean values of effective concentrations to inhibit mycelial growth by 50% (EC_{50} values) for the new fungicides ethaboxam, fluopicolide, mandipropamid, oxathiapiprolin, were 0.017 to 0.069 μg/ml (mean 0.035 μg/ml), 0.046 to 0.330 μg/ml (mean 0.133 μg/ml), 0.003 to 0.011 μg/ml (mean 0.005 μg/ml), 0.0002 to 0.0007 μg/ml (mean 0.0004 μg/ml), respectively. In comparison, the

EC_{50} value range for mefenoxam was 0.023 to 0.138 μg/ml (mean 0.061 μg/ml) and that for potassium phosphite was 12.9 to 361.2 μg/ml (mean 81.5 μg/ml). Greenhouse soil inoculation trials with 8-month-old Zutano seedlings and 10-month-old Dusa® and PS.54 clonal rootstocks were conducted to assess the efficacy of these fungicides for managing PRR. Mefenoxam and potassium phosphite were effective treatments, however, oxathiapiprolin, fluopicolide, and mandipropamid were more effective. Oxathiapiprolin reduced PRR incidence and pathogen population size in the soil by >90%, and plant shoot growth and root dry weight were significantly increased compared with the control and was one of the best treatments overall. The high activity and performance of these new fungicides supports their registrations on avocado for use in rotation and mixture programs, including with previously registered compounds, to reduce the risk of development and spread of resistance in pathogen populations.

INTRODUCTION

Phytophthora root rot (PRR) caused by *Phytophthora cinnamomi* Rands is the most important disease of avocado and limits production in California, Florida, and other locations worldwide. *P. cinnamomi* kills feeder roots and can also cause trunk cankers, resulting in reduced fruit yield and often tree death (Hardham 2005). PRR historically affected 60 to 75% of California avocado growers, causing losses of $40 million annually (Coffey 1987). Major expenditures for managing PRR also include cost and application of fungicides. Favorable conditions for spread and proliferation of the pathogen are wet, poorly drained soils at a wide range of temperatures (Erwin and Ribeiro 1996). The main infection propagules of *P. cinnamomi* are zoospores that are chemotactically attracted to the roots of plants (Zentmyer 1961). Chlamydospores, long-term resting structures, enable *P. cinnamomi* to persist in the soil for many years making it nearly impossible to completely eliminate the pathogen once the soil is infested (Mccarren et al. 2005).

Avocado PRR management includes the use of resistant rootstocks, proper irrigation practices, and chemical treatments (Coffey 1987). Commercially available, moderately resistant rootstocks include Dusa®, Toro Canyon, Duke 7, Steddom, Uzi, and Zentmyer (Bender et al. 2004). Among them, Dusa® is the current California industry standard enabling growers to cultivate avocado in *P. cinnamomic*-infested soil and maintain production. However, resistance of this rootstock is challenged by a new clonal group of more virulent *P. cinnamomi* isolates recently identified in California (Belisle et al. 2018; Pagliaccia et al. 2013). Cultural management practices include mulching,

gypsum application, and proper irrigation (Coffey 1987) using water sources not contaminated with propagules of *P. cinnamomi* (Mircetich et al. 1985). The pathogen has a very broad host range and is capable of infecting more than 5,000 plant species (Kamoun et al. 2015; Zentmyer 1980). Thus, even with the best management program, the pathogen can be re-introduced into an orchard from other plants or with irrigation and run-off water.

At present, the only fungicides available to control PRR of avocado are phosphonate-based (e.g., potassium phosphite; Fungicide Resistance Action Committee or FRAC Code P07, formerly Code 33) (FRAC 2018) and phenylamide compounds (e.g., mefenoxam; FRAC Code 4). Mefenoxam is an R-enantiomer of metalaxyl that was introduced in 1977. It has been effectively used for managing diseases caused by *Phytophthora* spp. and other Oomycota organisms (Hu et al. 2010). It is strongly inhibitory to mycelial growth and sporulation of these organisms due to interfering with RNA polymerases and blocking RNA synthesis (Müller and Gisi 2012) to these organisms. The risk of phenylamide resistance development is considered high due to a single-site mode of action (Gisi and Sierotzki 2015), and resistance has developed in *P. infestans*, *P. citricola*, *P. megasperma*, and *P. nicotianae* only few years after metalaxyl and mefenoxam became available for use (Coffey et al. 1984; Ferrin and Kabashima 1991; Gisi and Sierotzki 2015; Hwang and Benson 2005; Stack and Millar 1985). Little information is available on the sensitivity of *P. cinnamomi* populations to mefenoxam (Benson and Grand 2000; Duan et al. 2008; Hu et al. 2010), and no information is

available for isolates from avocado in California where this fungicide is mostly used by the nursery industry.

Phosphorous acid and its ionized compounds (i.e., phosphites) belong to the phosphonate group of fungicides. The specific mode of action of phosphite is largely unknown, but direct inhibition of pathogen growth (Dobrowolski et al. 2008; Fenn and Coffey 1984; Ma and McLeod 2014; Ouimette and Coffey 1989) and induction of the host plant defense system appear to be involved (Eshraghi et al. 2014; Groves et al. 2015; King et al. 2010). Reduced *in vitro* sensitivity to potassium phosphite in several *Phytophthora* spp., including *P. capsici*, *P. cinnamomi*, *P. citrophthora*, *P. infestans*, and *P. syringae* has been reported (Adaskaveg et al. 2017; Cohen and Samoucha 1984; Veena et al. 2010; Wilkinson et al. 2001). Potassium phosphite is the preferred PRR control treatment by avocado growers because it is less expensive than mefenoxam. Its optimal application by trunk injection, however, can be labor-intensive and costly, and furthermore, injection sites provide entry points for insect pests.

New Oomycota fungicides with different modes of action from mefenoxam and phosphonate fungicides have become available in recent years. Ethaboxam, a thiazole carboxamide (FRAC Code 22), disrupts microtubule organization in Oomycota (Uchida et al. 2005). Fluopicolide is a pyridinylmethyl-benzamide (FRAC Code 43) that disrupts cell division and mitosis by acting on spectrin-like proteins (Briggs et al. 2006; Jiang et al. 2015). Mandipropamid is a carboxylic acid amide (CAA; FRAC Code 40) fungicide targeting the pathogen cellulose synthase gene that is involved in cell wall biosynthesis (Gisi and Sierotzki 2015). Oxathiapiprolin, a piperidinyl-thiazole-isoxazoline (FRAC

Code 49), targets the oxysterol-binding protein of Oomycota organisms (Miao et al. 2016a).

The goal of this study was to determine whether the new Oomycota fungicides could be used to manage PRR of avocado. Thus, the objectives were to establish baseline sensitivities of a large number of isolates of *P. cinnamomi* representing the current pathogen population on avocado in California, compare these sensitivities to those of mefenoxam and potassium phosphite, and evaluate the efficacy of the four new fungicides as compared to the two registered ones for the management of PRR of avocado seedlings and clonal rootstocks in greenhouse studies.

MATERIALS AND METHODS

Isolates of *P. cinnamomi* used in this study. A total of 71 *P. cinnamomi* isolates were obtained from avocado roots and rhizosphere soil from northern (Los Angeles, Santa Barbara, and Ventura counties) and southern (Riverside and San Diego counties) avocado growing regions in California (Table 3.1). Fifty-three isolates were identified previously, including 32 isolates from an earlier study (Pagliaccia et al. 2013), and 18 isolates were identified in this study. Isolates were maintained as agar plugs in water during the study, and long-term in liquid nitrogen. The pathogen was cultured on 10% clarified V8 (V8C) agar (Ribeiro 1978).

The identity of the 18 recently recovered isolates was confirmed by sequencing the rDNA internal transcribed spacer (ITS) region (Cooke et al. 2000; Cooke and Duncan

1997). Mycelial DNA was extracted using the Qiagen DNeasy plant mini kit (Qiagen, Germantown, MD). Universal primers ITS1 and ITS4 (White et al. 1990) were used in PCR reactions. Each 25-µl reaction contained 2 µl of DNA (50 ng/µl), 2.5 µl of 10× PCR buffer (New England Biolabs, Ipswich, MA), 200 µM dNTPs, 0.4 µM of each primer, and 1.25 units of Taq DNA polymerase (New England Biolabs). PCR reactions were performed using a Programmed Thermal Controller (T100; Bio-Rad Laboratories, Hercules, CA) with conditions as follows: 95°C for 5 min; followed by 35 cycles at 95°C for 1 min, 50°C for 1 min, extension at 68°C for 1 min; and a final extension at 68°C for 10 min. PCR products were visualized in ethidium bromide-stained 1% agarose gels. PCR products were treated with Zymo DNA Clean & Concentrator (Zymo Research Corp, Irvine, CA) to remove excess primers and nucleotides, and sequenced in both directions (Eurofins Genomics, Louisville, KY). Consensus sequences were generated using BioEdit (version 7.2.5) (Hall 1999) and submitted to BLASTn searches of the GenBank nucleotide database (http://www.ncbi.nlm.nih.gov/).

Fungicides used. Fungicides used included ethaboxam (Intego; Valent USA, Walnut Creek, CA), fluopicolide (Presidio; Valent USA), mandipropamid (Revus; Syngenta Crop Protection, Greensboro, NC), oxathiapiprolin (Orondis OD; Syngenta Crop Protection), mefenoxam (Ridomil Gold SL; Syngenta Crop Protection), and potassium phosphite (ProPhyt; Helena Chemical, Collierville, TN) (Table 3.2).

***In vitro* fungicide sensitivities of *P. cinnamomi* isolates.** The sensitivity of mycelial growth of *P. cinnamomi* to potassium phosphite was tested using an agar dilution method. Potassium phosphite was amended to 10% V8C agar to obtain final

concentrations of 0 (control), 5, 25, 50, 100, 150, 300, or 600 μg/ml. Each triplicated petri dish for each concentration was inoculated in the center with a mycelial agar plug (6 mm in diameter) from 7- to 8-day-old cultures. After incubation in the dark at 22°C for 3 days, radial colony growth was measured. Mycelial growth percent inhibition of each isolate was calculated as compared with the control. This experiment was done twice. Effective concentrations to inhibit 50% of mycelial growth (EC_{50} values) were determined as described previously (Adaskaveg et al. 2015).

The sensitivity of mycelial growth to ethaboxam, fluopicolide, mandipropamid, mefenoxam, and oxathiapiprolin was evaluated using the spiral gradient dilution (SGD) method (Förster et al. 2004; Gray et al. 2018). Isolates were grown on sterile hydrophilic cellophane strips (5.5 cm × 0.5 cm; du Pont de Nemours, Wilmington, DE) that were placed onto V8C agar. Mycelium-covered agar strips of one-week-old cultures were placed between the cellophane strips. Plates were then incubated in the dark at 22°C for 7 to 9 days until mycelia had grown evenly over the cellophane strips. Aqueous stock solutions of ethaboxam (50 μg/ml), fluopicolide (100 μg/ml), mandipropamid (10 μg/ml), mefenoxam (50 μg/ml), or oxathiapiprolin (5 μg/ml), or water (control) were plated onto 15-cm V8C agar plates using a spiral plater (Autoplate 4000; Spiral Biotech, Norwood, MA) set at the exponential mode. Mycelium-covered cellophane strips were radially placed across the fungicide concentration gradient on the SGD plates. For each isolate, two strips were placed on opposite sides of duplicated plates, and the experiment was repeated. SGD plates were incubated in the dark at 22°C for 2 days. The location where

mycelial growth was inhibited by 50% was determined, and local fungicide concentrations (EC_{50} values) were calculated using the SGE software (Spiral Biotech).

Efficacy of Oomycota fungicides to control PRR of avocado seedlings and clonal rootstocks in greenhouse studies. Six- to eight-month-old avocado (*Persea americana* Mill. Zutano) seedlings and ten-month-old clonal rootstocks, Dusa® and PS.54 (Westfalia Technological Services, Tzaneen, South Africa), were obtained from local nurseries. Plants were transplanted into 19-liter pots using UC-A soil mix (Matkin and Chandler 1957). The average air temperature was between 25 and 28°C, and the relative humidity was between 40 and 50% during the experiments in the greenhouse. Plants were fertilized (Peters Excel 21-5-20; Scotts Miracle-Gro, Marysville, OH) once at the beginning of the studies and watered three times a week throughout the study.

Plants were inoculated using *P. cinnamomi*-colonized millet seeds (Drenth and Sendall 2001). For this, 175 ml sterile distilled water was added to 144 g of millet seeds in a 500-ml flask, and flasks were autoclaved for 30 min on two consecutive days. Ten plugs (6 mm in diameter) of a 4-day-old *P. cinnamomi* culture were added to each flask, and flasks were incubated in the dark at 25°C for 21 days. Equal proportions of seeds colonized by isolates 2109, 2117, or 2120 (obtained from avocado roots or rhizosphere soil in San Diego and Riverside counties) were mixed to prepare the final inoculum, and 4.4 g of inoculum was added to the soil around the base of the stem of each plant.

Fungicides were applied as a soil drench one week after inoculation. Two rates of fluopicolide, mandipropamid, and oxathiapiprolin were applied to seedlings. Only the high rates of fluopicolide, mandipropamid, oxathiapiprolin, and one rate of ethaboxam

were applied to two rootstocks; and one rate of each mefenoxam and potassium phosphite was applied to seedlings and rootstocks (Table 3.2). Fungicide application rates used for citrus (Hao et al. 2019) were used to calculate greenhouse rates for avocado based on the ratio of soil surface area of a tree in the field to a potted plant. Seedlings and rootstocks were arranged in a randomized complete block design (RCBD) with ten or six single-pot replicates, respectively. Fungicides (50 ml) were applied to the soil around the plant in each pot. Water (500 ml) was added to each pot immediately after application to move the fungicide into the soil. Inoculated plants treated with water were used as controls.

The efficacy of fungicide treatments was evaluated based on PRR incidence and pathogen population sizes in rhizosphere soil 16 to 17 weeks after fungicide applications. Rhizosphere soil was collected, and the root ball of each plant was rinsed with water. Feeder roots were cut into 1-cm-long pieces using a sterilized razor blade, and 20 pieces were placed onto each of two plates of *Phytophthora*-selective medium PARHFB-V8C (Ferguson and Jeffers 1999; Hao et al. 2018). When present, root pieces with discoloration were selected. Plates were incubated at 22°C for 2 to 3 days in the dark. *P. cinnamomi* colonies were identified by the distinctive coralloid-type mycelium with abundant hyphal swellings (Erwin and Ribeiro 1996), and representative colonies were sub-cultured and verified for species identity using species-specific TaqMan qPCR (Bilodeau et al. 2014). PRR incidence was calculated as the percentage of *P. cinnamomi*-infected root pieces of the total pieces plated.

For enumeration of soil populations, 10 g of rhizosphere soil from each plant was mixed with 90 ml sterile distilled water in a 250-ml Erlenmeyer 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 30 min. Aliquots of 1 ml soil suspension were spread onto two plates of PARHFB-V8C agar per plant. Plates were rinsed with water after 24 h at 22°C in the dark, incubated for another 2 to 3 days in the dark, and *P. cinnamomi* colonies were enumerated. *Phytophthora* propagule populations were calculated as CFU per gram of soil.

Dry weights of shoots of Zutano seedlings and roots of all plants were determined after drying at 50°C for 5 days. The experiment was conducted twice using avocado seedlings and repeated on two clonal rootstocks, Dusa® and PS.54.

Statistical analysis. Mean EC_{50} values for each isolate and fungicide to inhibit mycelial growth were analyzed using frequency histograms. The standard deviation was calculated from log_{10}-transformed EC_{50} values for ethaboxam, fluopicolide, mandipropamid, mefenoxam, oxathiapiprolin, and potassium phosphite. For each fungicide, the mean standard deviation of all isolates was used to calculate EC_{50} category bin widths as described by Scott (1979) using the following equation:

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

where h = the bin width of each EC_{50} category group, s = the mean standard deviation of all isolates, and n = the number of isolates tested. The number of isolates in each bin was determined, and bins were graphed in frequency histograms over the EC_{50} range.

For comparisons of the toxicity of the six fungicides for all isolates and for isolates from southern and northern growing regions, EC_{50} values were log_{10}-transformed, variances were analyzed using Bartlett's test of homogeneity, and

homogenous data sets were combined. Mean EC_{50} values for each fungicide for all isolates and for isolates from each growing region were compared using analysis of variance (ANOVA) for balanced datasets. For comparing EC_{50} values of fungicides, isolates from the two growing regions, and their interaction, a two-way analysis was done using general linear model (GLM) procedures for unbalanced datasets and Tukey's studentized range test for multiple mean comparisons with a 95% family-wise confidence level.

For repeated greenhouse experiments, the homogeneity of variances was tested using Bartlett's test of homogeneity. Homogenous data sets were combined and analyzed using ANOVA, and differences between means of treatments were determined using Fisher's least significant difference (LSD) test. ANOVA, GLM, homogeneity, and mean separation analyses were performed in SAS (version 9.4; SAS Institute, Inc., Cary, NC). Results were considered significant at $P \leq 0.05$.

RESULTS

Identification of isolates. ITS sequences of 18 recently collected isolates were 784 to 799 bp long. BLASTn searches showed that all had a 99% similarity to reference sequences of *P. cinnamomi*. Representative sequences were deposited in GenBank (accession numbers MH842130, MH842131, MH842132, MH842133, MH842134, MH842135, and MH842136 for isolates 346, 345, 344, 343, 341, 338, and 337, respectively).

***In vitro* fungicide sensitivities of *P. cinnamomi* isolates.** All 71 isolates evaluated were highly sensitive to ethaboxam, fluopicolide, mandipropamid, oxathiapiprolin, and mefenoxam. EC_{50} value ranges (means) for the six fungicides were 0.017 to 0.069 μg/ml (0.035 μg/ml), 0.046 to 0.330 μg/ml (0.133 μg/ml), 0.003 to 0.011 μg/ml (0.005 μg/ml), 0.0002 to 0.0007 μg/ml (0.0004 μg/ml), 0.023 to 0.138 μg/ml (0.061 μg/ml), and 12.9 to 361.2 μg/ml (81.5 μg/ml) for ethaboxam, fluopicolide, mandipropamid, oxathiapiprolin, mefenoxam, and potassium phosphite, respectively (Table 3.3). Differences in the range of sensitivities for ethaboxam, fluopicolide, mandipropamid, oxathiapiprolin, and mefenoxam were 4.1-, 7.2-, 3.7-, 3.5-, and 6.0-fold, respectively. For potassium phosphite, a wider sensitivity range with a 28-fold difference between the least and the most sensitive isolate was observed. The mean EC_{50} values for all isolates were significantly different for each fungicide and for the isolates collected within each of the two regions (Table 3.3). Oxathiapiprolin had the lowest mean EC_{50} value of 0.0004 μg/ml for all isolates evaluated.

There was a significant interaction ($P < 0.001$) of fungicides and geographical origin of isolates in the two-way GLM analysis. Mean EC_{50} values for isolates from southern and northern growing regions were not significantly different for ethaboxam ($P = 0.362$), fluopicolide ($P = 0.637$), mandipropamid ($P = 0.217$), oxathiapiprolin ($P = 0.053$), and mefenoxam ($P = 0.866$). For potassium phosphite, however, mean values for isolates from southern (i.e., 98.9 μg/ml) growing regions were significantly ($P = 0.001$) higher than those from northern (i.e., 47.3 μg/ml) regions (Table 3).

Frequency histograms of EC_{50} values for ethaboxam, fluopicolide, mandipropamid, oxathiapiprolin, and mefenoxam exhibited a unimodal distribution of sensitivities for the isolates tested (Fig. 3.1A to E). In contrast, the frequency histogram for potassium phosphite showed a bimodal distribution representing populations of isolates with different EC_{50} value ranges (Fig. 3.2). Thirty-eight isolates had values in a range from 12.9 to 59.7 μg/ml (mean 24.5 μg/ml), whereas the remaining 33 isolates exhibited a reduced sensitivity with EC_{50} values in a range from 79.1 to 361.2 μg/ml (mean 147.0 μg/ml).

Isolates 2109, 2117, and 2120 that were used for inoculations in the greenhouse studies had EC_{50} values for ethaboxam of 0.057 μg/ml, 0.048 μg/ml, and 0.060 μg/ml, for fluopicolide of 0.125 μg/ml, 0.106 μg/ml, and 0.099 μg/ml, for mandipropamid of 0.004 μg/ml, 0.004 μg/ml, 0.003 μg/ml, for oxathiapiprolin of 0.0003 μg/ml, 0.0002 μg/ml, and 0.0002 μg/ml, for mefenoxam of 0.055 μg/ml, 0.061 μg/ml, and 0.068 μg/ml, and 0.003 μg/ml, and for potassium phosphite of 116.7 μg/ml, 120.0 μg/ml, and 165.3 μg/ml, respectively.

Efficacy of Oomycota fungicides to control PRR of Zutano avocado seedlings in greenhouse studies. All fungicide treatments evaluated significantly ($P < 0.05$) reduced PRR incidence and *P. cinnamomi* propagule populations in rhizosphere soil compared with the untreated infected control (Fig. 3.3). Oxathiapiprolin at the high rate of 0.028 g/pot resulted in significantly the lowest incidence of PRR with a 95% reduction from the untreated control where 82% of the plated root pieces were found to be colonized by *P. cinnamomi* (Fig. 3.3A). The lower rate of this fungicide was also very

effective and was statistically similar to either rate of fluopicolide. The low rate of fluopicolide (0.028 g/pot) performed similar to either rate of mandipropamid, whereas mefenoxam and potassium phosphite were the least effective with reductions in PRR incidence from the control of 51 and 43%, respectively. Oxathiapiprolin at either rate most effectively reduced the number of viable *P. cinnamomi* propagules in the soil compared with all other treatments (Fig. 3.3B). All other treatments significantly reduced pathogen populations as compared with the control, and there were no significant differences among these latter fungicides (Fig. 3.3B).

Variances of shoot and root dry weights were not homogenous between experiments according to Bartlett's test ($P = 0.030$ and $P = 0.013$ for shoot and root dry weight, respectively), thus, data are presented for each experiment (Table 3.4). Similar trends were observed for most of the treatments in the two experiments. In both studies, all treatments significantly increased shoot dry weight as compared with the untreated control. Oxathiapiprolin at both rates and fluopicolide at the high rate in both experiments, and mandipropamid at the high rate in the second experiment had significantly ($P < 0.05$) the highest shoot growth. Shoot dry weight of these treatments was increased between 192% (i.e., mandipropamid high rate – first experiment) and 265.5% (i.e., fluopicolide high rate – first experiment) as compared with the untreated control. Root dry weight of inoculated plants was highest after using oxathiapiprolin at either rate or fluopicolide at the high rate in both experiments, mandipropamid at the high rate in the second experiment, or fluopicolide at the low rate in the first experiment. Increases as compared to the control ranged from 192.8% (i.e., mandipropamid high rate

– second experiment) to 306.5% (i.e., fluopicolide low rate – first experiment). Root dry weight was not significantly different as compared with the control after potassium phosphite treatment in the first experiment.

Efficacy of Oomycota fungicides to control PRR of clonal avocado rootstocks. Only the higher of the two concentrations of fluopicolide, mandipropamid, and oxathiapiprolin from the seedling studies, and ethaboxam were evaluated in experiments with clonal rootstocks and compared with mefenoxam and potassium phosphite. Untreated plants of both rootstocks developed a similar incidence of PRR (i.e., 75.4% on Dusa® and 80% on PS.54). All treatments significantly reduced the incidence of PRR compared with the untreated controls on both rootstocks (Fig. 3.4A, C). On Dusa®, a significant difference among treatments was only observed between oxathiapiprolin with 2.1% incidence and potassium phosphite with 16.3% incidence. The other fungicides were all intermediate in efficacy. On PS.54, oxathiapiprolin resulted in the lowest incidence of PRR (i.e., 12.9%), and this was statistically similar to fluopicolide (21.7% incidence). Ethaboxam at the rate used was the least effective of the fungicides with 51.7% incidence.

Pathogen propagules were effectively reduced by all treatments on the Dusa® rootstock with no significant difference among treatments (Fig. 3.4B). Still, mandipropamid, oxathiapiprolin, mefenoxam, and potassium phosphite reduced soil populations to zero levels. In contrast, on the PS.54 rootstock, only oxathiapiprolin significantly reduced *P. cinnamomi* populations in the soil (Fig. 3.4D).

Among the fungicides tested, all except mefenoxam significantly ($P \leq 0.05$) increased root dry weight of inoculated PS.54 plants from that of control (Table 3.5). On Dusa® rootstock, there was no significant difference in root dry weight among the six fungicides evaluated, but only ethaboxam-treated plants had a significantly higher root dry weight as compared with the control.

DISCUSSION

In this study, the four new Oomycota-targeting fungicides ethaboxam, fluopicolide, mandipropamid, and oxathiapiprolin demonstrated high *in vitro* toxicity with relatively low mean EC_{50} values to the avocado root rot pathogen *P. cinnamomi*. The *in vitro* sensitivities for each of these compounds displayed a unimodal distribution and a narrow range of EC_{50} values for mycelial growth inhibition of 71 isolates representing the current *P. cinnamomi* population in major avocado growing areas in California. The narrow ranges in sensitivities among isolates with no distinct less-sensitive outliers in the distribution may suggest a reduced potential for selection of resistance with the proper use of these fungicides. Because *P. cinnamomi* isolates were never previously exposed to ethaboxam, fluopicolide, mandipropamid, and oxathiapiprolin, the sensitivity ranges reported herein can be referred to as baseline distributions that can be used as references in future monitoring for fungicide resistance in populations of the pathogen.

In our study, oxathiapiprolin had the lowest EC_{50} values for all isolates among the new fungicides evaluated ranging from 0.0002 to 0.0007 µg/ml. This fungicide also was shown to be highly inhibitory to other *Phytophthora* spp. from a wide range of hosts by others with mean EC_{50} values of less than 0.001 µg/ml (Belisle et al. 2018; Ji and Csinos 2015; Martin et al. 2014; Miao et al. 2016b; Qu et al. 2016). Similarly, Gray et al. (2018) found that oxathiapiprolin had the lowest range of EC_{50} values of 0.0002 to 0.0015, 0.0002 to 0.0003, 0.0003 to 0.001, and <0.0003 µg/ml for *P. citrophthora*, *P. syringae*, *P. nicotianae*, and *P. hibernalis*, respectively, as compared with the other three compounds. Together, reported inhibitory values for oxathiapiprolin are generally 10- to 1000-fold lower than those for ethaboxam, fluopicolide, mandipropamid, and mefenoxam, depending on the fungicide-species combination. Thus, the *in vitro* toxicity of oxathiapiprolin to *P. cinnamomi* from avocado reported in our study is lower than for any previous fungicide evaluated against this pathogen.

EC_{50} values for fluopicolide, mandipropamid, and ethaboxam for *P. cinnamomi* in our study were also within the range of values previously determined for several other *Phytophthora* spp. (Belisle et al. 2018; Gray et al. 2018; Jackson et al. 2010; Jiang et al. 2015; Kim et al. 2004; Qu et al. 2016; Saville et al. 2015). The range of EC_{50} values for mefenoxam in our study (0.023 to 0.138 µg/ml) was similar to that previously reported for *P. cinnamomi* from avocado (Belisle et al. 2018), Fraser fir (Benson and Grand 2000), and woody ornamentals (Duan et al. 2008; Hu et al. 2010) in the United States. Thus, the current usage pattern for this fungicide to control avocado PRR in California nurseries and orchards has not resulted in mefenoxam resistance in *P. cinnamomi* populations.

In contrast to the other fungicides, a wide range of *in vitro* sensitivities was detected for potassium phosphite, and there was a significant difference in mean EC_{50} values between isolates from the two geographical regions, confirming a previous report (Belisle et al. 2018). The higher value for isolates from southern California production areas may be due to higher field rates or more frequent applications of potassium phosphite to manage PRR in avocado orchards. The bimodal distribution for the 71 isolates in this study separates the current pathogen population into two sensitivity groups indicating a shift in population sensitivity. A baseline for this compound, however, was never established before commercial field usage. Still, prolonged use of phosphite caused a shift toward reduced sensitivity of *P. cinnamomi* isolates from avocado orchards in Australia (Dobrowolski et al. 2008) and South Africa (Ma and McLeod 2014). Phosphonate (e.g., fosetyl-Al) resistance has also been reported for *P. cinnamomi* from *Chamaecyparis lawsoniana* in nurseries (Vegh et al. 1985), downy mildew of lettuce (Brown et al. 2004), and recently in *P. citrophthora, P. nicotianae,* and *P. syringae* from citrus in California (Adaskaveg et al. 2017). With direct and indirect effects on the pathogen, the resistance potential of potassium phosphite is considered relatively low (Dobrowolski et al. 2008). The extensive and often sole use of this FRAC group in California avocado orchards to combat PRR (Coffey et al. 1984; Darvas and Becker 1984), however, is expected to eventually lead to resistance.

In our greenhouse studies, avocado seedlings and rootstocks were inoculated with *P. cinnamomi* isolates from southern avocado production areas that have been described as more virulent (Belisle et al. 2018). A high incidence of PRR developed on untreated

control plants of seedlings and both rootstocks with more than 75% of plated root pieces colonized by the pathogen. The high incidence on the Dusa® rootstock that is considered more tolerant to PRR is likely due to our selection of discolored root pieces for plating of all samples. The four new fungicides were moderately to highly effective in reducing PRR and *P. cinnamomi* populations in rhizosphere soil of the avocado seedlings and rootstocks used. Overall, oxathiapiprolin was the most effective among fungicides evaluated. In experiments with Zutano seedlings, the efficacy of oxathiapiprolin at the low rate of 70 g/Ha was 2- to 33-times higher than that of the other fungicides and 2- to 4-times higher than that of mandipropamid, a CAA fungicide. In a study on managing *P. capsici* on peppers (Miao et al. 2016b), the difference in effectiveness of oxathiapiprolin at 30 g/Ha as compared to the CAA dimethomorph at 262.5 g/Ha was similar to our study using the same FRAC codes of fungicides. In response to reducing PRR, avocado plants treated with oxathiapiprolin generally developed more shoot and root growth as compared with untreated plants.

On the avocado seedlings and rootstocks used, fluopicolide, mandipropamid, and ethaboxam treatments also effectively reduced the incidence of PRR compared with the control. *P. cinnamomi* propagules in the rhizosphere soil were only significantly reduced on the Zutano seedlings and the Dusa® rootstock. These latter treatments were often significantly more effective than potassium phosphite or mefenoxam; whereas fluopicolide often performed statistically similar to oxathiapiprolin. Still, the efficacy of potassium phosphite was demonstrated with significant reductions in PRR on the seedlings and rootstocks although its overall performance may have been compromised

by the use of three *P. cinnamomi* isolates with reduced sensitivities to the fungicide (i.e., EC_{50} values between 116.7 μg/ml and 165.3 μg/ml) in our soil inoculations. These results also could explain why potassium phosphite is still effectively used in managing PRR in California since many growers cultivate avocado trees grafted on the Dusa® rootstock.

Thus, highly effective alternatives to mefenoxam and the phosphonates were identified by us for the management of avocado PRR. Oxathiapiprolin used at low rates provided similar or better efficacy than the other fungicides. Oxathiapiprolin, fluopicolide, mandipropamid, and ethaboxam previously demonstrated high efficacy against selected foliar and root diseases of vegetable and tree crops caused by Oomycota organisms in greenhouse and field studies. Thus, the four fungicides were highly efficacious in reducing Phytophthora root rot of citrus caused by *P. nicotianae* and *P. citrophthora* (Hao et al. 2019). Oxathiapiprolin, fluopicolide, and mandipropamid were more effective in managing *P. capsici* on watermelon than mefenoxam or potassium phosphite (Kousik et al. 2011, 2016). In other studies, oxathiapiprolin was shown to be highly effective in managing diseases of vegetable crops caused by *Phytophthora* species including *P. capsici* (Ji and Csinos 2015; Miao et al. 2016b) and *P. infestans* (Pasteris et al. 2016) and controlled black shank of tobacco caused by *P. nicotianae* (Bittner and Mila 2016; Ji et al. 2014). Ethaboxam was shown to be an effective treatment for tomato late blight (*P. infestans*), as well as Phytophthora blight (*P. capsici*) of pepper (Kim et al. 2004).

Based on our studies, registration of oxathiapiprolin for use on avocado has been initiated through the Inter-regional Research Project No. 4 (IR-4), and ethaboxam,

fluopicolide, and mandipropamid are proposed for further development on avocado. Additional evaluations will have to be done under field conditions using rootstocks with different growth characteristics and susceptibilities to PRR. The availability of fungicides with new modes of action and options for rotation and mixture programs using previously registered and new fungicides will help reduce the risk of development and spread of resistance in *P. cinnamomi* populations in California avocado production. Growers currently rely heavily on the use of phosphonate-based fungicides, and as we demonstrated, pathogen populations are shifting towards reduced sensitivity to this fungicide class. Thus, there is an urgent need to register fungicides with new modes of action. In our greenhouse studies, overall treatment efficacy in reducing PRR and soil inoculum levels of the pathogen on the susceptible PS.54 was reduced as compared with the more tolerant Dusa® rootstock, indicating additive effects of fungicide use and rootstock selection. In an integrated approach for a durable and effective management of PRR that allows the continued economical production of avocados in *P. cinnamomi*-infested soils, the use of tolerant rootstocks is critical along with irrigation management and cultural practices such as using mulching and planting in areas with good soil drainage.

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REFERENCES

1. Adaskaveg, J. E., Förster, H., Hao, W., and Gray, M. 2017. Potassium phosphite resistance and new modes of action for managing Phytophthora diseases of citrus in the United States. Pages 205-210 in: Modern Fungicides and Antifungal Compounds. H. B. Deising, B. Fraaije, A. Mehl, E. C. Oerke, H. Sierotzki, and G. Stammler, eds. Vol. VIII. Deutsche Phytomedizinische Gesellschaft, Braunschweig, Germany.

2. Adaskaveg, J. E., Hao, W., and Förster, H. 2015. Postharvest strategies for managing Phytophthora brown rot of citrus using potassium phosphite in combination with heat treatments. Plant Dis. 99:1477-1482.

3. Belisle R., McKee, B., Hao, W., Crowley, M., Arpaia, M. L., Miles, T. D., Dr. James E. Adaskaveg, J. E., and Manosalva, P. 2018. Phenotypic characterization of genetically distinct *Phytophthora cinnamomi* isolates from avocado. Phytopathology 10.1094/PHYTO-09-17-0326-R.

4. Bender, G. S., Menge, J. A., and Arpaia, M. L. 2004. Avocado rootstocks. Pages 45-53 in: Avocado Production in California: A Cultural Handbook for Growers. Book 1: Background Information, 2nd ed. G. S. Bender ed. University of California Cooperative Extension, San Diego County and The California Avocado Society, Oakland, CA.

5. Benson, D. M., and Grand, L. F. 2000. Incidence of Phytophthora root rot of Fraser fir in North Carolina and sensitivity of isolates of *Phytophthora cinnamomi* to metalaxyl. Plant Dis. 84:661-664.

6. Bilodeau, G. J., Martin, F. N., Coffey, M. D., and Blomquist, C. L. 2014. Development of a multiplex assay for genus- and species-specific detection of *Phytophthora* based on differences in mitochondrial gene order. Phytopathology 104:733-748.

7. Bittner, R. J., and Mila, A. L. 2016. Effects of oxathiapiprolin on *Phytophthora nicotianae*, the causal agent of black shank of tobacco. Crop Prot. 81:57-64.

8. Briggs, G., Mansfield, D., Moloney, B., Gary, S., and Wegmann, T. 2006. The discovery and chemistry of fluopicolide: A new standard for oomycetes disease control. Pflanzenschutz Nachrichten Bayer 59:141-152.

9. Brown, S., Koike, S. T., Ochoa, O. E., Laemmlen, F., and Michelmore, R. W. 2004. Insensitivity to the fungicide fosetyl-aluminum in California isolates of the

lettuce downy mildew pathogen, *Bremia lactucae*. Plant Dis. 88:502-508.

10. Coffey, M. D. 1987. *Phytophthora* root rot of avocado - an integrated approach to control in California. California Avocado Society Yearbook 71:121-137.

11. Coffey, M. D., Klure, L. J., and Bower, L. A. 1984. Variability in sensitivity to metalaxyl of isolates of *Phytophthora cinnamomi* and *Phytophthora citricola*. Phytopathology 74:417-422.

12. Cohen, Y., and Samoucha, Y. 1984. Cross-resistance to 4 systemic fungicides in metalaxyl-resistant strains. Plant Dis. 68:137-139.

13. Cooke, D. E., and Duncan, J. M. 1997. Phylogenetic analysis of *Phytophthora* species based on ITS1 and ITS2 sequences of the ribosomal RNA gene repeat. Mycol. Res. 101:667-677.

14. Cooke, D. E., Drenth, A., Duncan, J. M., Wagels, G., and Brasier, C. M. 2000. A molecular phylogeny of *Phytophthora* and related oomycetes. Fungal Genet. Biol. 30:17-32.

15. Darvas, J. M., and Becker, O. 1984. Failure to control *Phytophthora cinnamomi* and *Pythium splendens* with metalaxyl after its prolonged use. South African Avocado Growers' Association Yearbook 7:77-78.

16. Dobrowolski, M. P., Shearer, B. L., Colquhoun, I. J., O'Brien, P. A., and Hardy, G. E. 2008. Selection for decreased sensitivity to phosphite in *Phytophthora cinnamomi* with prolonged use of fungicide. Plant Pathol. 57:928-936.

17. Drenth, A., and Sendall, B. 2001. Inoculation of soil. Pages 37-38 in: Practical guide to detection and identification of *Phytophthora*. CRC for Tropical Plant Protection, Brisbane, Australia.

18. Duan, C. H., Riley, M. B., and Jeffers, S. N. 2008. Characterization of *Phytophthora cinnamomi* populations from ornamental plants in South Carolina, USA. Arch. Phytopathol. Plant Protect. 41:14-30.

19. Erwin, D. C., and Ribeiro, O. K. 1996. Phytophthora Diseases Worldwide. APS Press, The American Phytopathological Society, St. Paul, MN.

20. Eshraghi, L., Anderson, J. P., Aryamanesh, N., Mccomb, J. A., Shearer, B. L., and Hardy, G. E. St. J. 2014. Defence signalling pathways involved in plant resistance and phosphite-mediated control of *Phytophthora cinnamomi*. Plant Mol. Biol. Rep. 32:342-356.

21. Fenn, M. E., and Coffey, M. D. 1984. Studies on the *in vitro* and in vivo antifungal activity of fosetyl-A1 and phosphorous acid. Phytopathology 74:606-611.

22. Ferguson, A. J., and Jeffers, S. N. 1999. Detecting multiple species of Phytophthora in container mixes from ornamental crop nurseries. Plant Dis. 83:1129-1136.

23. Ferrin, D. M., and Kabashima, J. N. 1991. *In vitro* insensitivity to metalaxyl of isolates of *Phytophthora citricola* and *P. parasitica* from ornamental hosts in southern California. Plant Dis. 75:1041-1044.

24. FRAC. 2018. FRAC Code List: Fungicides sorted by mode of action. Online publication. http://www.frac.info.

25. Förster, H., Kanetis, L., and Adaskaveg, J. E. 2004. Spiral gradient dilution, a rapid method for determining growth responses and 50% effective concentration values in fungus-fungicide interactions. Phytopathology 94:163-170.

26. Gray, M., Hao, W., Forster, H., and Adaskaveg, J. 2018. Baseline sensitivities of new fungicides and their toxicity to selected life stages of *Phytophthora* species from citrus in California. Plant Dis. 102:734-742.

27. Gisi, U., and Sierotzki, H. 2015. Oomycete fungicides: phenylamides, quinone outside inhibitors, and carboxylic acid amides. Pages 145-174 in: Fungicide Resistance in Plant Pathogens. H. Ishii and D. W. Hollomon, eds. Springer, Tokyo, Japan.

28. Groves, E., Howard, K., Hardy, G., and Burgess, T. 2015. Role of salicylic acid in phosphite-induced protection against Oomycetes; a *Phytophthora cinnamomi-Lupinus augustifolius* model system. Eur. J. Plant Pathol. 141:559-569.

29. Hall, T. A. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl. Acids. Symp. Ser. 41:95-98.

30. Hao, W., Miles, T. D., Martin, F. N., Browne, G., Förster, H., and Adaskaveg, J. E. 2018. Temporal occurrence and niche preferences of *Phytophthora* species causing brown rot of citrus in the Central Valley of California. Phytopathology 108:384-391.

31. Hao, W., Gray, M. A., Förster, H., and Adaskaveg, J. E. 2019. Evaluation of new Oomycota fungicides for management of Phytophthora root rot of citrus in California. Plant Dis. 10.1094/PDIS-07-18-1152-RE.

32. Hardham, A. R. 2005. *Phytophthora cinnamomi*. Mol. Plant Pathol. 6:589-604.

33. Hu, J., Hong, C., Stromberg, E. L., and Moorman, G. W. 2010. Mefenoxam sensitivity in *Phytophthora cinnamomi* isolates. Plant Dis. 94:39-44.

34. Hwang, J., and Benson, D. M. 2005. Identification, mefenoxam sensitivity, and compatibility type of *Phytophthora* spp. attacking floriculture crops in North Carolina. Plant Dis. 89:185-190.

35. Jackson, K. L., Yin, J., Csinos, A. S., and Ji, P. 2010. Fungicidal activity of fluopicolide for suppression of *Phytophthora capsici* on squash. Crop Prot. 29:1421-1427.

36. Ji, P. and Csinos, A. S. 2015. Effect of oxathiapiprolin on asexual life stages of *Phytophthora capsici* and disease development on vegetables. Ann. Appl. Biol. 166: 229-235.

37. Ji, P., Csinos, A. S., Hickman, L. L., and Hargett, U. 2014. Efficacy and application methods of oxathiapiprolin for management of black shank on tobacco. Plant Dis. 98:1551-1554.

38. Jiang, L., Wang, H., Xu, H., Qiao, K., Xia, X. and Wang, K. 2015. Transportation behaviour of fluopicolide and its control effect against *Phytophthora capsici* in greenhouse tomatoes after soil application. Pest. Manag. Sci. 71: 1008-1014.

39. Kamoun, S., Furzer, O., Jones, J. D. G., Judelson, H. S., Ali, G. S., Dalio, R. J., Roy, S. G., Schena, L., Zambounis, A., Panabières, F., Cahill, D., Ruocco, M., Figueiredo, A., Chen, X. R., Hulvey, J., Stam, R., Lamour, K., Gijzen, M., Tyler, B. M., Grünwald, N. J., Mukhtar, M. S., Tomé, D. F., Tör, M., Van Den Ackerveken, G. , McDowell J., Daayf, F., Fry, W. E., Lindqvist-Kreuze. H., Meijer, H. J., Petre, B., Ristaino, J., Yoshida, K., Birch, P. R., and Govers, F. 2015. The top 10 oomycete pathogens in molecular plant pathology. Mol. Plant Pathol. 16:413-434.

40. Kim, D.-S., Chun, S.-J., Jeon, J.-J., Lee, S.-W., and Joe, G.-H. 2004. Synthesis and fungicidal activity of ethaboxam against Oomycetes. Pest. Manag. Sci. 60:1007-1012.

41. King, M., Reeve, W., Van der Hoek, M. B., Williams, N., McComb, J., O'Brien, P. A., and Hardy, G. E. St. J. 2010. Defining the phosphite-regulated transcriptome of the plant pathogen *Phytophthora cinnamomi*. Mol. Genet. Genomics 284:425-435.

42. Kousik, C. S., Adams, M. L., Jester, W. R., Hassell, R., Harrison, H. F., and Holmes, G. J. 2011. Effect of cultural practices and fungicides on Phytophthora fruit rot of watermelon in the Carolinas. Crop Prot. 30:888-894.

43. Kousik, C., Egel, D., Ji, P., and Quesada-Ocampo, L. 2016. Fungicide rotation schemes and Melcast for managing Phytophthora fruit rot of watermelon in Southeastern United States. (Abstr.) Phytopathology 106:S4.68-69.

44. Ma, J., and McLeod, A. 2014. *In vitro* sensitivity of South African *Phytophthora cinnamomi* to phosphite at different phosphate concentrations. South African Avocado Growers' Association Yearbook 37:79-84.

45. Martin, F. N, Blair, J. E., and Coffey, M. D. 2014. A combined mitochondrial and nuclear multilocus phylogeny of the genus Phytophthora. Fungal Genet. Biol. 66:19-32.

46. Matkin, O. A., and Chandler, P. A. 1957. The U.C. type soil mixes. Pages 68-85 in: The U.C. System for Producing Healthy Container-Grown Plants. K. F. Baker, ed. University of California division of Agriculture and Natural Resources, Manual 23, Berkeley, CA.

47. Mccarren, K., Mccomb, J. A., Shearer, B., and Hardy, G. 2005. The role of chlamydospores of *Phytophthora cinnamomi* - A review. Australas. Plant Pathol. 34:333-338.

48. Miao, J., Cai, M., Dong, X., Liu, L., Lin, D., Zhang, C., Pang, Z., and Liu, X. 2016a. Resistance assessment for oxathiapiprolin in *Phytophthora capsici* and the detection of a point mutation (G769W) in PcORP1 that confers resistance. Front Microbiol. *7*:615.

49. Miao, J, Dong, X, Lin, D., Wang, Q., Liu, P, Chen, F., Du, Y., and Liu, X. 2016b. Activity of the novel fungicide oxathiapiprolin against plant-pathogenic oomycetes. Pest. Manag. Sci. 72:1572-1577.

50. Mircetich, S. M., Browne, G. T., Krueger, W., and Schreader W. 1985. *Phytophthora* spp. isolated from surface water irrigation sources in California. (Abstr.) Phytopathology 75:1346.

51. Müller, U., and Gisi, U. 2012. Newest aspects of nucleic acid synthesis inhibitors: Metalaxyl-M. Pages 901-908 in: Modern Crop Protection Compounds, 2nd ed. W. Krämer, U. Schirmer, P. Jeschke, and M. Witschel eds. Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim, Germany.

52. Ouimette, D. G., and Coffey, M. D. 1989. Comparative antifungal activity of four

phosphonate compounds against isolates of nine *Phytophthora* species. Phytopathology 79:761-767.

53. Pagliaccia, D., Pond, E., McKee, B., and Douhan, G. W. 2013. Population genetic structure of *Phytophthora cinnamomi* associated with avocado in California and the discovery of a potentially recent introduction of a new clonal lineage. Phytopathology 103:91-97.

54. Pasteris, R. J., Hanagan, M. A., Bisaha, J. J., Finkelstein, B. L., Hoffman, L. E., Gregory, V., Andreassi, J. L., Sweigard, J. A., Klyashchitsky, B. A., Henry, Y. T., and Berger, R. A. 2016. Discovery of oxathiapiprolin, a new oomycete fungicide that targets an oxysterol binding protein. Bioorg. Med. Chem. 24:354-361.

55. Qu, T., Shao, Y., Csinos, A. S., and Ji, P. 2016. Sensitivity of *Phytophthora nicotianae* from tobacco to fluopicolide, mandipropamid, and oxathiapiprolin. Plant Dis. 100:2119-2125.

56. Ribeiro, O. K. 1978. A Source Book of the Genus *Phytophthora*. J. Cramer, Vaduz, Liechtenstein.

57. Saville, A., Graham, K., Grünwald, N. J., Myers, K., Fry, W. E., and Ristaino, J. B. 2015. Fungicide sensitivity of U.S. genotypes of *Phytophthora infestans* to six oomycete-targeted compounds. Plant Dis. 99:659-666.

58. Stack, J., and Millar, R. 1985. Isolation and characterization of a metalaxyl-insensitive isolate of *Phytophthora megasperma* f. sp. *medicaginis*. Phytopathology 75:1387-1392.

59. Scott, D. W. 1979. On optimal and data-based histograms. Biometrika 66:605-610.

60. Uchida, M., Roberson, R. W., Chun, S.-J. and Kim, D.-S. 2005. *In vivo* effects of the fungicide ethaboxam on microtubule integrity in *Phytophthora infestans*. Pest. Manag. Sci. 61:787-792.

61. Veena, S. S., Anandaraj, M., and Sarma, Y. R. 2010. Variability in the sensitivity of *Phytophthora capsici* isolates to potassium phosphonate. Indian Phytopathol. 63:71-75.

62. Vegh, I., Leroux, P., LeBerre, A., and Lanen, C. 1985. Detection sur *Chamaecyparis lawsoniana* 'Elwoodii' d'une souche de *Phytophthora cinnamomi*. Rands resistante au phosethyl-Al. Revue Horticole 262:19-21.

63. White, T. J., Bruns, T., Lee, S., and Taylor, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. Pages 315-322 in: PCR Protocols: A Guide to Methods and Applications. M. A. Innis, J. J. Gelfand, J. J. Sninsky, and T. J. White, eds. Academic Press, San Diego, CA.

64. Wilkinson, C. J., Shearer, B. L., Jackson, T. J., and Hardy, G. E. S. 2001. Variation in sensitivity of Western Australian isolates of *Phytophthora cinnamomi* to phosphite *in vitro*. Plant Pathol. 50:83-89.

65. Zentmyer, G. A. 1961. Chemotaxis of zoospores for root exudates. Science 19:1595-1596.

66. Zentmyer, G. A. 1980. *Phytophthora cinnamomi* and the Diseases it Causes. Monograph No. 10. APS Press, St. Paul, MN.

Table 3.1. Isolates of *Phytophthora cinnamomi* from California used in this study

No. of isolates	Year collected	County of origin	Source
34	2004, 2005, 2008, 2009, 2015, 2017	San Diego	Avocado roots, rhizosphere
12	2009, 2011, 2015. 2016	Riverside	Avocado roots, rhizosphere
1	2015	Los Angeles	Avocado roots, rhizosphere
13	1996, 2009, 2010, 2017	Ventura	Avocado roots, rhizosphere
10	2010, 2017	Santa Barbara	Avocado roots, rhizosphere
1	Unknown	Unknown	Avocado roots

Table 3.2. Fungicides and application rates used in this study

				Applicate rate	
Active ingredient	**Product name**	**Registrant**	**FRAC code[x]**	**Field (g / Ha)[y]**	**Greenhouse (g / pot)[z]**
Ethaboxam 34.2%	Intego	Valent USA	22	702	0.140
Fluopicolide 39.5%	Presidio	Valent USA	43	140	0.028
				210	0.042
Mandipropamid 23.3%	Revus	Syngenta Crop	40	146	0.029
		Protection		292	0.058
Mefenoxam 45.3%	Ridomil Gold	Syngenta Crop	4	561	0.112
	SL	Protection			
Oxathiapiprolin 9.4%	Orondis OD	Syngenta Crop	49	70	0.014
		Protection		140	0.028
Potassium phosphite	ProPhyt	Helena	33	2353	0.469
54.5%		Chemical			

[x] FRAC 2018.

[y] Registrant-recommended field application rates for tree crops (Hao et al. 2019) were used to calculate greenhouse rates for avocado. Field rates per tree were calculated based on 346 trees/Ha.

[z] Greenhouse rates per pot were calculated from field rates per tree based on the soil area per tree:soil area per pot = 14.5:1.

Table 3.3. *In vitro* sensitivity ranges and mean values of effective concentrations to inhibit mycelial growth of 71 isolates of *Phytophthora cinnamom*i by 50% (EC_{50} values) for the fungicides used in this study

	All isolates (n=71)			Southern isolates (n=46)[w]			Northern isolates (n=25)[x]			Southern vs. northern means[y]
Fungicide	**Range**	**Mean**	**Tukey[z]**	**Range**	**Mean**	**Tukey**	**Range**	**Mean**	**Tukey**	***P*-Value**
Ethaboxam	0.017-0.069	0.035	d	0.018-0.066	0.034	d A	0.017-0.069	0.037	d A	0.362
Fluopicolide	0.046-0.330	0.133	b	0.046-0.330	0.131	b A	0.069-0.257	0.135	b A	0.637
Mandipropamid	0.003-0.011	0.005	e	0.003-0.011	0.005	e A	0.003-0.011	0.006	e A	0.217
Oxathiapiprolin	0.0002-0.0007	0.0004	f	0.0002-0.0006	0.0003	f A	0.0002-0.0007	0.0004	f A	0.053
Mefenoxam	0.023-0.138	0.061	c	0.026-0.138	0.061	c A	0.023-0.100	0.062	c A	0.866
Potassium phosphite	12.9-361.2	81.5	a	12.9-316.2	98.9	a A	16.6-266.2	47.3	a B	0.001

[w] Isolates collected in southern avocado growing areas of California (Riverside and San Diego counties).

[x] Isolates collected in northern avocado growing areas of California (Los Angeles, Santa Barbara, and Ventura counties).

[y] Mean EC_{50} values were analyzed using a general linear model for analysis of variance and Tukey's studentized range test for multiple mean comparisons with 95% family-wise confidence level. Values followed by the same uppercase letter are not significantly

different ($P > 0.05$) between southern and northern isolates for each fungicide in each row.

[z] Mean EC_{50} values were analyzed using an analysis of variance and Tukey's studentized range test for multiple mean comparisons with 95% family-wise confidence level. Values followed by the same lowercase letter are not significantly different ($P > 0.05$) from each other in each column.

Table 3.4. Effect of new Oomycota fungicides on shoot and root dry weights of Zutano avocado seedlings inoculated with *Phytophthora cinnamomi* in greenhouse studies

	1st experiment		2nd experiment	
Treatment (g / pot)	**Shoot DW[y] (g)**	**Root DW (g)**	**Shoot DW (g)**	**Root DW (g)**
Control	31.9 c[z]	9.3 e	46.0 e	16.6 f
Fluopicolide (0.028)	79.1 a	28.5 a	80.6 bc	30.4 bcde
Fluopicolide (0.042)	84.7 a	25.7 ab	94.9 a	34.9 ab
Mandipropamid (0.029)	56.5 b	20.2 bcd	77.4 bc	29.3 cbc
Mandipropamid (0.058)	58.9 b	17.6 cd	88.6 ab	32.0 abcd
Mefenoxam (0.112)	48.6 b	17.6 cd	73.8 cd	25.3 e
Oxathiapiprolin (0.014)	76.4 a	24.4 abc	96.8 a	33.3 abc
Oxathiapiprolin (0.028)	81.5 a	24.4 abc	94.4 a	36.4 a
Potassium phosphite (0.469)	53.1 b	15.7 de	62.8 d	26.6 de

[y] Dry weight (DW) of shoots and roots of each plant was measured after drying at 50°C for 5 days.

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

Table 3.5. Effect of new Oomycota fungicides on root dry weights of Dusa® and PS.54 avocado rootstocks inoculated with *Phytophthora cinnamomi* in greenhouse studies

Treatment (g / pot)	Root DW (g)	
	Dusa®	**PS.54**
Control	20.1 b	16.2 c
Ethaboxam (0.140)	25.4 a	28.6 ab
Fluopicolide (0.042)	22.2 ab	28.1 ab
Mandipropamid (0.058)	20.4 b	28.5 ab
Mefenoxam (0.112)	22.6 ab	22.6 bc
Oxathiapiprolin (0.028)	23.5 ab	31.4 a
Potassium phosphite (0.469)	22.3 ab	30.2 ab

[y] Root dry weight (DW) of each plant was measured after drying the samples at 50°C for 5 days.

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

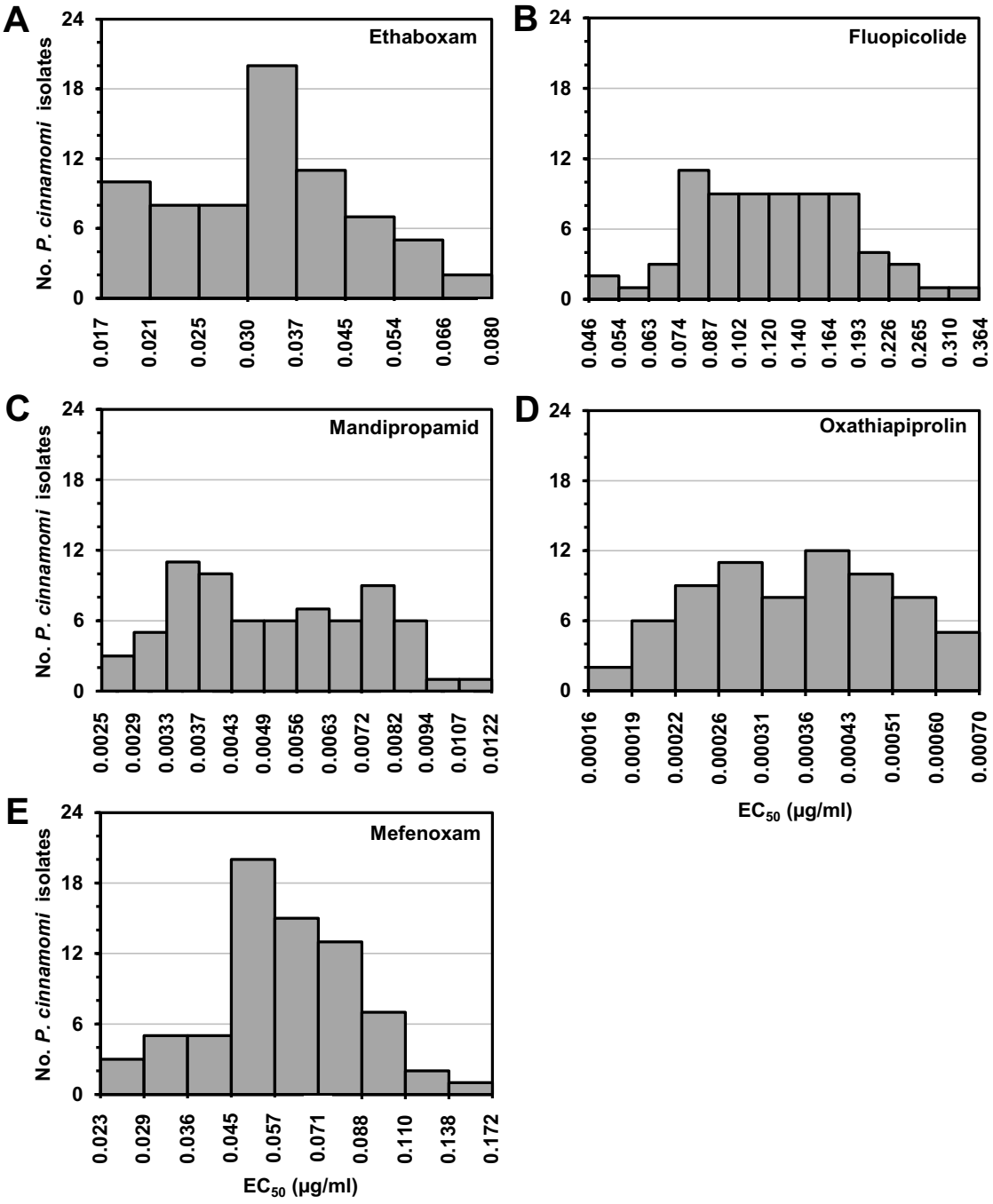

Fig. 3.1. Frequency histograms of effective concentrations to inhibit mycelial growth of 71 isolates of *Phytophthora cinnamomi* by 50% (EC_{50} values) for **A,** ethaboxam, **B,** fluopicolide, **C,** mandipropamid, **D,** oxathiapiprolin, and **E,** mefenoxam as determined by

the spiral gradient dilution method. Bar height represents the number of isolates within each bin, and bin widths were calculated using Scott's method (Scott 1979).

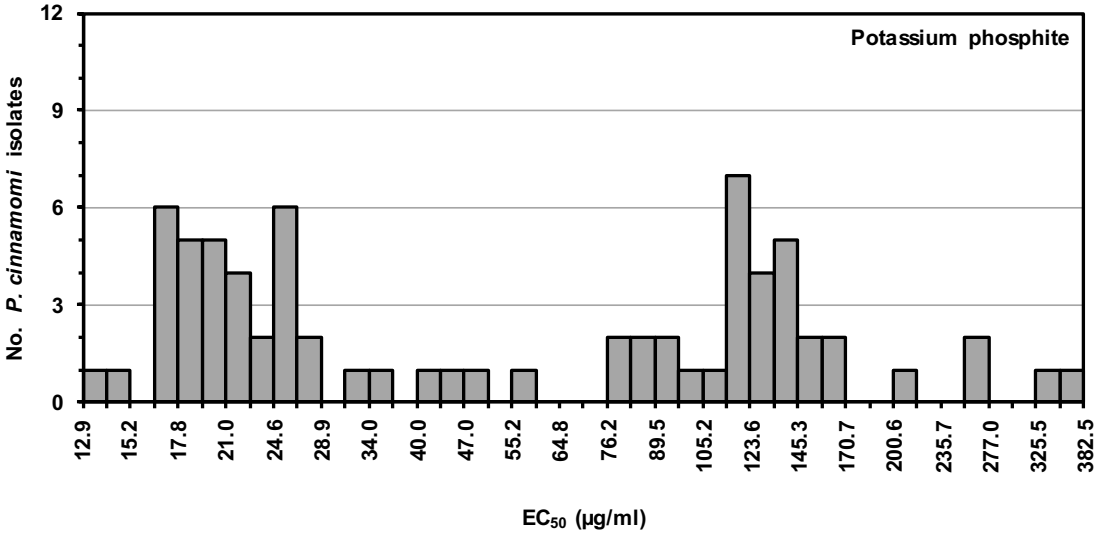

Fig. 3.2. Frequency histogram of effective concentrations to inhibit mycelial growth of 71 isolates of *Phytophthora cinnamomi* by 50% (EC_{50} values) for potassium phosphite as determined by the agar dilution method. Bar height represents the number of isolates within each bin, and bin widths were calculated using Scott's method (Scott 1979).

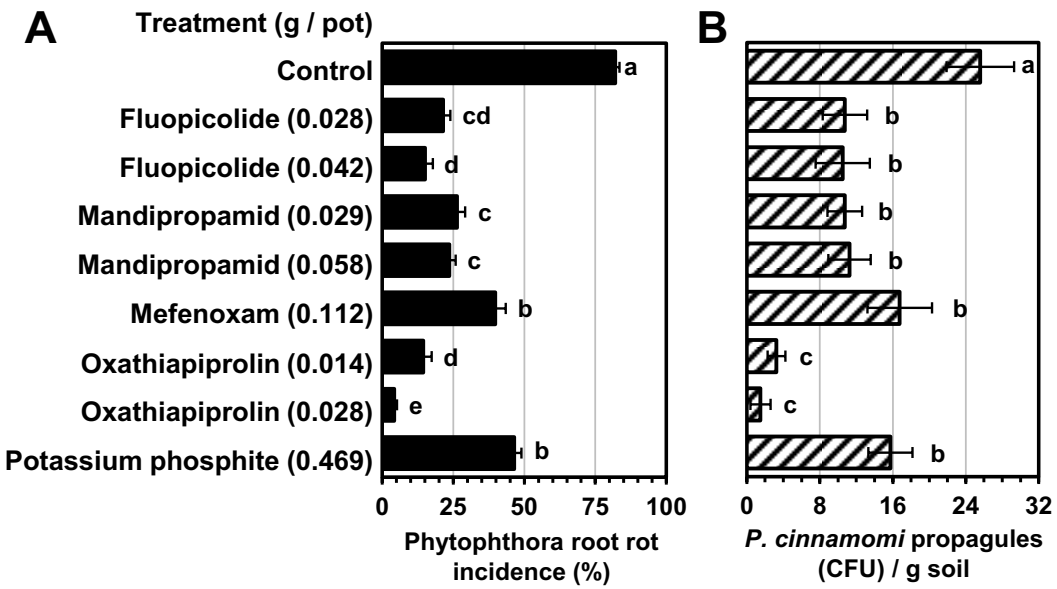

Fig. 3.3. Efficacy of new Oomycota fungicides to manage **A,** Phytophthora root rot of Zutano avocado seedlings and **B,** rhizosphere populations of *Phytophthora cinnamomi* under greenhouse conditions. Bars followed by the same letter are not significantly different according to Fisher's least significance difference test at $P > 0.05$. Standard error bars are also shown.

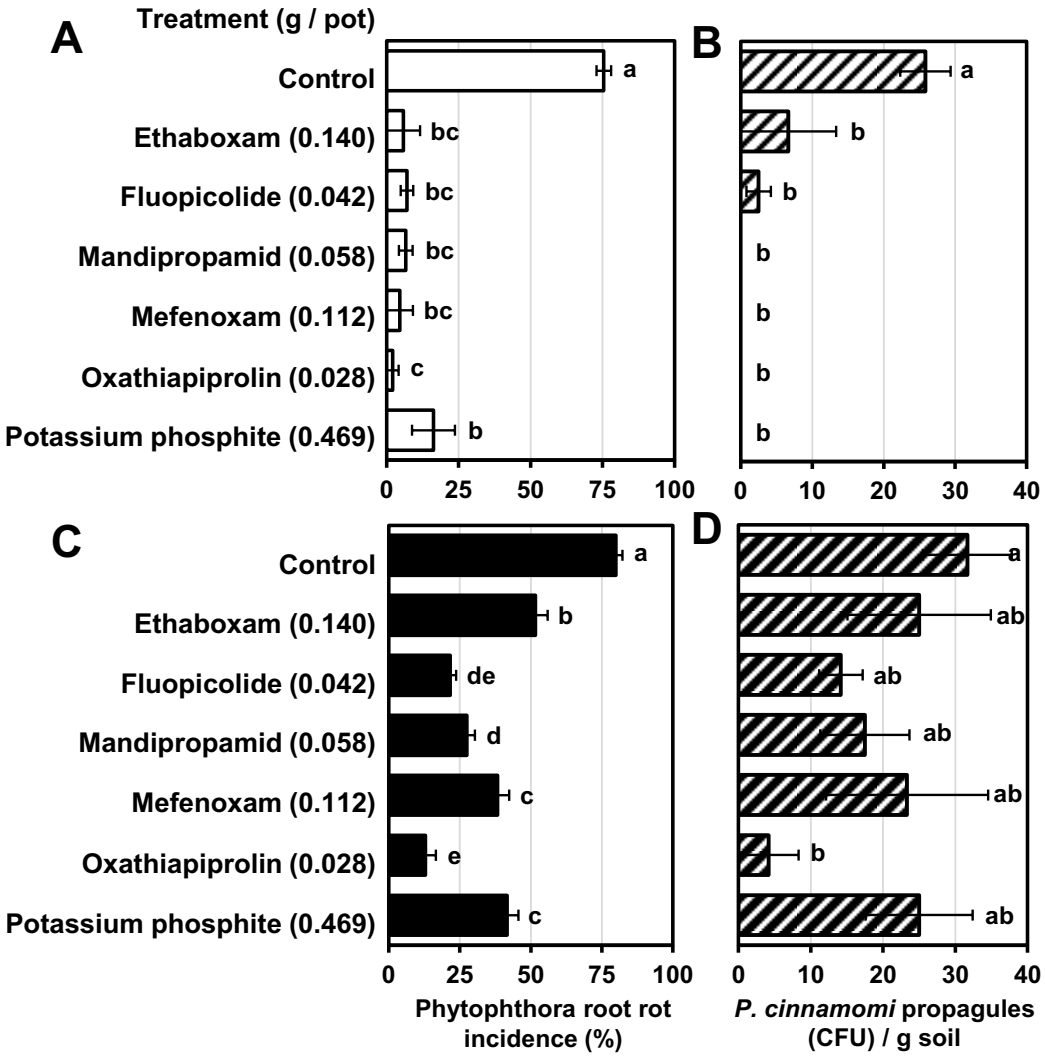

Fig. 3.4. Efficacy of new Oomycota fungicides to manage **A, C,** Phytophthora root rot and **B, D,** rhizosphere populations of *Phytophthora cinnamomi* of clonal avocado rootstock **A, B,** Dusa® and **C, D,** PS.54 under greenhouse conditions. Bars followed by the same letter are not significantly different according to Fisher's least significance difference test at $P > 0.05$. Standard error bars are also shown.

CHAPTER IV. RNASEQ TRANSCRIPTOME ANALYSIS OF THE MODEL PLANT *NICOTIANA BENTHAMIANA* AFTER *PHYTOPHTHORA CINNAMOMI* INFECTION

Belisle, R. J., Xu, G., Jackson, N., Shands, A., McKee, B., and Manosalva, P. 2018. RNAseq transcriptome analysis of the model plant *Nicotiana benthamiana* after *Phytophthora cinnamomi*. Gene (*in preparation*).

ABSTRACT

Phytophthora Root Rot (PRR) of avocado, caused by *Phytophthora cinnamomi,* is the most limiting disease of avocado worldwide. Despite the importance of this pathogen, the molecular and genetic basis of plant-*P. cinnamomi* interactions are largely unknown. Functional genomics studies to identify genes associated with resistance are challenging in avocado due to limitations associated with tree biology and lack of reference genomes and efficient transformation systems (Pliego-Alfaro and Litz 2007). To circumvent these limitations, we established a new *Nicotiana benthamiana-P. cinnamomi* pathosystem and developed a detached leaf assay (Belisle et al. 2018), which coupled to RNAseq analysis and high-quality reference genomes, allow us to conduct functional studies of candidate genes in this pathosystem. *N. benthamiana* has been widely used to study plant-*Phytophthora* interactions since it can be used for transient silencing and overexpression to study plant and pathogen gene functions. Our *N. benthamiana-Pc* pathosystem was

developed to identify candidate genes associated with virulence and plant genes associated with resistance. Defense responses against *P. cinnamomi* in this model plant were similar to those reported in avocado and other hosts after infection. Genes involved in hormone signaling, receptor-like kinases, NBS-LRRs, transcription factors, secondary metabolism and production of antimicrobial compounds, and pathogenesis-related (PR) were differentially expressed (DE) after pathogen infection. The differential expression after pathogen infection of several of these candidate genes were validated in *N. benthamiana* and avocado using a detached leaf assay. Functional validation of one WRKY transcription factor, which was up-regulated after *P. cinnamomi* infection was done by transient expression in *N. benthamiana*. Overexpression of this transcription factor in this model plant enhanced resistance to *P. cinnamomi*. The identification of candidate genes from host plants associated with resistance can then be modified or used to generate resistant avocado rootstock varieties using biotechnology or through traditional breeding. The generation of molecular markers linked to genes conferring resistance to *P. cinnamomi* can be also used for an accurate genomic selection at avocado seedling stages using Marker Assisted Selection (MAS).

INTRODUCTION

Plant pathogenic oomycetes fall into two general categories when it comes to pathogenicity. There are Phytophthora species that can infect only one, or a few different hosts like *Phytophthora infestans* (Mont.) de Bary, and then there are species that can infect hundreds or even thousands of different plant species such as *P. cinnamomi* Rands (Hardham and Blackman 2018; Kamoun et al. 2015). *P. cinnamomi* is of particular interest in California because it causes Phytophthora root rot (PRR) of avocado, in fact, PRR is the most destructive disease of avocado production worldwide (Erwin and Ribeiro 1996). PRR limits production of avocado by killing feeder roots which reduces fruit yield and can cause tree death (Hardham 2005). *P. cinnamomi* impacts other fruit crops such as peach, pineapple, and highbush blueberry, as well as affecting natural stands of eucalyptus, pine, and oak (Shands et al. 2016; Zentmyer 1980). Areas that have become infested with *P. cinnamomi* will never completely remove this pathogen from the soil. Current chemical treatments are being challenged by the emergence of isolates that are more virulent and less sensitive to potassium phosphite (Belisle et al. 2018).

The current challenges of PRR treatment of avocado necessitates a better understanding of the molecular and genetic basis of plant-*P. cinnamomi* interactions. Taking advantage of the wide host range of *P. cinnamomi*, we developed a detached leaf assay in *Nicotiana benthamiana* to elucidate the molecular and genetic basis of plant immunity against *P. cinnamomi* (Belisle et al 2018). The hemibiotrophic lifestyle of *P. cinnamomi* was confirmed in this model system through differential staining and

quantitative PCR (qPCR) pathogen DNA quantification. The model plant, *N. benthamiana* (Bombarely et al. 2012), has been widely used to study the pathogenicity and virulence of similar broad range and root Phytophthora pathogens such as *P. capsici* (Chen et al. 2013; Vega-Arreguin et al. 2014), *P. palmivora* (Ekchaweng et al. 2017; Evangelisti et al. 2017; Goodin et al. 2008), and *P. parasitica* (Dalio et al. 2018; Robin and Guest 1994). Furthermore, several studies using model plants, crops, and tree crops to study pathogenicity, virulence, and fungicide efficacy of root rot pathogens such as *P. sojae*, *P. capsici*, *P. parasitica*, *P. palmivora*, *P. cinnamomi*, and *P. ramorum* have been performed using detached-leaf assays (Denman et al. 2005; Dong et al. 2015; Ekchaweng et al. 2017; Esharghi et al. 2011; Helliwell et al. 2016; Robin and Guest 1994; Vega-Arreguin et al. 2014). Using the tools developed in previous studies and combining them with RNAseq analysis as well as functional assays using this model plant it becomes possible to gain a better understanding of plant defense responses against *P. cinnamomi* infection.

Previous transcriptomic studies on avocado and model systems provides important information on plant gene expression in response to infection by *P. cinnamomi*. Avocado defense gene expression has been analyzed three separate times over the last eight years (Mahomed and Van den Berg 2011; Reeksting et al. 2014; Reeksting et al 2016). Mahomed and Van den Berg (2011) used the tolerant avocado rootstock Dusa® to study the gene expression changes after *P. cinnamomi* inoculation. By comparing expressed sequence tags and 454 pyrosequencing they were able to identify six defense related genes. The defense genes identified encoded: cytochrome P450-like TBP (TATA

box binding protein), thaumatin, PR10 (psemI), metallothionein-like protein, MLO transmembrane protein encoding gene, and a gene encoding a universal stress protein (Mahomed and Van den Berg 2011). In a follow up study, again on the resistant avocado rootstock Dusa® (Reeksting et al. 2014), 16 additional defense genes encoding: WRKY transcription factors, phenylalanine ammonia-lyase (PAL), beta-glucanase, allene oxide synthase, allene oxide cyclase, oxophytodienoate reductase, 3-ketoacyl CoA thiolase, F-box proteins, ethylene biosynthesis, isoflavone reductase, glutathione s-transferase, cinnamyl alcohol dehydrogenase, cinnamoyl-CoA reductase, cysteine synthase, quinone reductase, and NPR1 were differentially expressed after *P. cinnamomi* infection. Reeksting et al. (2016) found up-regulated transcripts corresponding to cytochrome P450, a germin-like protein (GLP), and chitinase genes after *P. cinnamomi* infection using microarray technology. It has been stated (Reeksting et al. 2014), that an important difference between gene expression in avocado and model systems is that the salicylic acid (SA) response is only seen in infected avocado, which is associated with a defense response to biotrophic and hemibiotrophic pathogens. It has been further asserted (Islam et al. 2018) that *P. cinnamomi* infection of model plants initiates the jasmonic acid and ethylene pathways associated with necrotrophic pathogens. Although there are differences between expression patterns in avocado and the numerous model plants that have been studied to better understand plant defense to *P. cinnamomi*, there are also many similarities.

Model plants used to better understand plant defense gene response to *P. cinnamomi* infection include; *Zea mays*, *Arabidopsis thaliana*, *Lupinus angustifolius*,

Castanea sativa (chestnut), *Eucalyptus nitens*, *Lomandra longifolia*, and most recently *N. benthamiana* (Allardyce et al. 2013; Belisle et al. 2018; Eshraghi et al. 2014; Islam et al. 2017; Meyer et al. 2016; Rooks et al. 2008; Santos et al. 2017). The gene expression in susceptible model hosts such as *L. angustifolius* and *N. benthamiana* can be compared to tolerant hosts like *A. thaliana* and *L. longifolia* to identify differences that may be associated with resistance to *P. cinnamomi*.

Santos et al (2017) compared the gene expression between a susceptible and resistant variety of chestnut. They found that genes encoding for proteins involved in pathogen recognition proteins (Cast_LRR-RLK and Cast_C2CD), were significantly up-regulated in the resistant variety especially before inoculation. Six out of eight defense related genes including; *WRKY31* and *LRR-RLK*'s were more highly expressed in the uninoculated *C. crenata* when compared to the uninoculated *C. sativa*. This increased basal defense to *P. cinnamomi* may contribute to this variety's resistance. Gene expression in *E. nitens* in response to *P. cinnamomi* infection included up-regulated over-represented gene ontology terms related to JA and ET signaling (Meyer et al. 2016). Interestingly, *pathogenesis-related gene 9* (PR-9) was down-regulated and represents a cross-species effector target during *P. cinnamomi* infection. Functional genomics and validation of these defense genes has only been performed in one study in *A. thaliana.* Eshraghi et al. (2014) reported that an auxin Arabidopsis mutant (*phr1-1*) was more susceptible to *P. cinnamomi* infection than the wild type indicating the role of auxin pathways in *P. cinnamomi* defenses.

The main challenges for the identification of *P. cinnamomi* resistance genes in avocado are the lack of tools available for functional genomic studies and limitations associated with tree crop biology. Next-generation sequencing has provided some information on the expression of defense-related genes in avocado infected with *P. cinnamomi*. However, the lack of the genome sequence and absence of functional genomic tools for avocado makes it difficult to determine and confirm their contributions to resistance against *P. cinnamomi*. The *N. benthamiana* model plant provides the opportunity to conduct functional genomic studies to determine the role of defense response genes to *P. cinnamomi* resistance that is not yet available in the avocado system or other tree hosts. Model plants including *A. thaliana* (Esharghi et al. 2011; Robinson and Cahill 2003), *L. angustifolius* (Esharghi et al. 2014; Gunning et al. 2013), and *Medicago truncatula* (Huisman et al. 2015) have been previously reported as susceptible hosts for this oomycete pathogen and have been used to study *P. cinnamomi* pathogenesis and plant responses to this pathogen. Although whole genome sequencing was available for these pathosystems, functional assays were not conducted with the exception of one study in *Arabidopsis* implicating the auxin signaling pathway with defense response against *P. cinnamomi* (Eshraghi et al. 2014).

Conducting RNAseq studies in *N. benthamiana* system at different time points during the infection process will provide a foot-hold into the defense gene expression pattern during *P. cinnamomi* infection and will allow us to conduct functional studies of selected defense genes using this *N. benthamiana–P. cinnamomi* pathosystem. Differentially expressed pathways and genes can be then validated by RT-qPCR i*n N.*

benthamiana and in avocado inoculated with *P. cinnamomi* using a detached leaf assay. Functional validation of the most promising genes can be done in *N. benthamiana* by transient overexpression or silencing to determine their contribution to *P. cinnamomi* resistance. If similar expression patterns are found in avocado it is reasonable to consider this gene a good candidate for marker assisted breeding or biotechnology in avocado. As genomic tools for avocado quickly become more available the methods developed in this system will become more applicable to this fruit tree crop. RNAseq analysis of infected *N. benthamiana* roots can complement this system by identifying what genes are universally expressed in the plant in response to *P. cinnamomi* infection and what gene expression is unique to the roots.

Functional genomics are lacking in avocado; therefore, the objectives of this study were i) to establish a model system to look at defense gene expression in response to *P. cinnamomi* infection, ii) validate differentially expressed defense genes using over-expression in the same *N. benthamiana* model system, and iii) establishing connections to similarly expressed defense genes in avocado in response to *P. cinnamomi* infection. This information will help to select candidate defense genes in avocado for marker assisted breeding or biotechnology.

MATERIALS AND METHODS

Isolates of *P. cinnamomi* used in this study. All of the *P. cinnamomi* isolates used in this study were obtained from the *P. cinnamomi* collection at the UCR Avocado

Rootstock Breeding Program. Isolates were maintained as agar plugs in water during the study (Boesewinkel 1976). Agar plugs were cultured on 10% clarified V8 (V8C) agar (Ribeiro 1978).

Plant materials used in this study. Avocado rootstocks Dusa® (moderate resistance to PRR; industry standard) and PS.54 (susceptible to PRR; Westfalia Technological Services, Tzaneen, South Africa) (Bender et al. 2004) were obtained from local nurseries. Six-month-old clonally propagated avocado plants were transplanted into 19-liter pots using UC-A soil mix (Matkin and Chandler 1957) after the nurse seed was removed. Plants were maintained in a greenhouse with an average air temperature between 25 and 28°C and the relative humidity between 40 and 50%. Plants were fertilized (Peters Excel 21-5-20; Scotts Miracle-Gro, Marysville, OH) once at the beginning of the studies and watered twice every week. *Nicotiana benthamiana* were seeded in trays with Sunshine #3 mix (Sun Gro Horticulture, Agawam, MA) and germinated seedlings were transplanted into 1-liter pots two weeks post germination. Plants were maintained in a temperature-controlled growth room at 22°C with a 16 h / 8 h light / dark cycle at 40 to 50% relative humidity.

Detached leaf inoculations. Five-week-old *N. benthamiana* were used for detached leaf inoculations as described in Manosalva et al. (2015) using *P. cinnamomi* zoospore suspensions. Newly expanded Dusa® and PS.54 leaves were used for the avocado detached leaf assays. *Z*oospores suspensions of *P. cinnamomi* isolate 2113 (obtained from avocado roots or rhizosphere soil in Santa Barbara County) were produced following the protocol described by Londsdale et al. (1988). In brief, 10 (7 mm

in diameter) plugs from 6-day-old 10% V8C agar cultures were placed on a sterile Miracloth (EMD Millipore Corp., Billerica, MA), which was previously placed on a 10-cm 10% V8C agar plate. Plates were incubated for 6 days at 22°C in the dark, and the Miracloth was removed from the plate and placed in a 500 ml flask with 100 ml of 10% V8C broth. After incubation at 22°C in the dark on a rotary shaker (MaxQ4000; Thermo Fisher Scientific, Waltham, MA) at 160 rpm for 20 h, the Miracloth was rinsed 3 times with a salt solution (Londsdale et al. 1988) and incubated for an additional 18 h at 22°C in the dark on the shaker. The Miracloth was rinsed with 100 ml of 18°C sterile deionized water (SDW) and incubated in 50 mL of 18°C SDW for 1.5 h to induce zoospore release. Zoospores were counted using a hemocytometer (Bright-Line, Horsham, PA.).

The abaxial surface of *N. benthamiana* and avocado leaves were inoculated by placing aliquots of 20 µl zoospore suspension at 1×10^4 zoospores/ml. The inoculated leaves were placed in 15 cm Petri dishes containing 50 ml 1.5% water agar (Difco agar; Becton Dickinson, Sparks, MD) and incubated in a precision plant growth incubator (Thermo Scientific, Waltham, MA) under a 6 h / 18 h light / dark cycle at 25°C. An equal number of mock inoculated leaves (SDW as inoculum) were prepared in the same manner. Three biological replicates for both inoculated and mock *N. benthamiana* leaves were prepared for 6, 12, 24, 36, and 48 h post inoculation (hpi) time points, and four replicates were included for both Dusa® and PS.54 rootstocks at 24 hpi.

RNA extraction. Total RNA was extracted from both inoculated and mock *N. benthamiana* leaves collected at 6, 12, 24, 36, and 48 hpi time points. Three leaf discs (9 mm in diameter) were cut using a #4 cork borer from each leaf and frozen in liquid

nitrogen immediately. RNA of each leaf disc was extracted using the RNeasy Plant Mini Kit (Qiagen, Germantown, MD) following the manufacturer's instructions and then treated with DNase using the Invitrogen TURBO DNA-free Kit (Thermo Fisher Scientific, Waltham, MA) to remove genomic DNA contaminations. RNA cleanup was performed using the Zymo RNA Clean and Concentrator Kit (Zymo Research Corp., Irvine, CA). The RNA integrity number of each sample was assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) using the Plant RNA Nano Assay. All RNA samples used had a RIN score of greater than 6.0.

RNAseq library construction and Illumina sequencing. Libraries for RNA sequencing were constructed for both inoculated and mock *N. benthamiana* leaf samples collected at 6, 12, and 24, 36, and 48 hpi by Novogene Corporation, Sacramento, CA. Briefly, Messenger RNA (mRNA) was enriched using oligo (dT) beads. The enriched mRNA was randomly fragmented in fragmentation buffer, followed by cDNA synthesis using random hexamers and reverse transcriptase. After first-strand synthesis, a custom second-strand synthesis buffer (Illumina) was added with dNTPs, RNAse H, and *Escherichia coli* polymerase I to generate the second strand by nick-translation. Three biological replicates were included for 6, 12, and 24 hpi time points, and 2 biological replicates were for 36 and 48 hpi. Libraries were sequenced using Illumina HiSeq™ platform by Novogene Corporation. Libraries corresponding to inoculated samples at 6 and 12 hpi were sequencing at 60 million reads and the rest of the libraries were done at 30 million reads.

Sequence data analysis. Bioinformatic analyses were conducted by Novogene. Briefly, raw reads were filtered to remove reads containing Illumina adapters or reads of low quality. Clean reads were mapped to the reference *N. benthamiana* genome (https://solgenomics.net/organism/Nicotiana_benthamiana/genome) (Bombarely et al. 2012) using TopHat2 (Kim et al. 2013). The gene expression level of each gene was based on the transcript abundance and calculated by normalizing to the fragments per kilobase of transcript sequence per million mapped reads (FPKM) value. HTSeq software with the union mode was used to analyze the gene expression levels in this experiment. Genes exhibiting statistical significance $P < 0.05$ and a $\log_2$ fold change ratio ≥ 1 or ≤ -1 were defined as differentially expressed genes (DEGs). Adjusted P values were obtained using a false discovery rate (FDR) calculation (Benjamini and Hochberg 1995).

Real time quantitative PCR (RT-qPCR) validation. RT-qPCR analysis of six differentially expressed plant defence genes were performed to validate RNAseq results. The six target genes included those encoding aristolochene synthase (Niben101Scf00700g00005.1) (EAS), cytochrome P450 (Niben101Scf00158g04018.1) (CYP), glutathione S-transferase (Niben101Scf03147g10015.1) (GST), phenylalanine ammonia-lyase (Niben101Scf05617g00005.1) (PAL), premnaspirodiene oxygenase (Niben101Ctg13347g00002.1) (CYP family of genes), and WRKY transcription factor 51 (Niben101Scf01942g04001.1) (WRKY51). Total RNA used for RT-qPCR (1 µg each sample) was DNase treated using the Invitrogen TURBO DNA-free Kit and reverse transcribed to cDNA using the Invitrogen Superscript III Kit (Thermo Fisher Scientific). Each 10-ul reaction included 2.5 µl of a cDNA dilution of 100 ng/µl, 5 µl iQ SYBR

Green Supermix (Bio-Rad Laboratories, Inc., Hercules, CA), 0.5 µM of each forward and reverse qPCR primers (Table 4.1). Reactions were amplified using a CFX384 Touch Bio-Rad Real Time PCR system (Bio-Rad Laboratories) at the following conditions: 95°C for 3 min (initial denaturation) followed by 44 cycles of denaturation at 95°C for 10 s and annealing/extension at 55°C for 45 s. A dissociation curve was generated at the end of each qPCR to verify single product amplification. Gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ (Ct = cycle threshold) comparative method. Eukaryotic translation elongation factor 1 (EF1-α1) was used as an endogenous reference gene for *N. benthamiana*. The results were reported as the mean ± standard error of three biological replications. Pearson's correlation test was used to evaluate the correlation between the RNAseq and qPCR techniques.

Plasmid construction. Full length CDS of the *WRKY51* gene of *N. benthamiana* (Niben101Scf01942g04001.1) was amplified using the primers in Table 4.1 using conventional PCR using a Programmed Thermal Controller (PTC-100; MJ Research, Inc., Watertown, MA) with conditions as follows: 95°C for 5 min; followed by 35 cycles at 95°C for 1 min, 50°C for 1 min, and 68°C for 1 min; and a final extension at 68°C for 10 min. Each PCR reaction contained 2 µl of DNA (50 ng/µl), 2.5 µl of 10× PCR buffer (New England Biolabs, Ipswich, MA), 200 µM dNTPs, 0.4 µM of each primer, and 1.25 units of Taq DNA polymerase (New England Biolabs). PCR products were confirmed on 1% agarose gel (Bio-Rad Laboratories), stained with ethidium bromide (0.05% v/v), and visualized under UV light using a Universal Hood UV Light Table (75s/00124 With Camera Model Rs170s; Bio-Rad Laboratories). PCR products were cleaned using Zymo

DNA Clean and Concentrator Kit, A-Tailed (dATP (10mM), 1.25 units of Taq DNA polymerase, and 5 µl 10× PCR buffer), gel purified (1% agarose gel), and cloned into the pXSN-HA plasmid (Chen et al. 2009) using the *Xcm*I restriction site. Ligation reaction consisted of pXSN-HA *Xcm*I-digested plasmid (1 µl), PCR insert (8 µl), T4 ligase enzyme (0.5 µl) and T4 buffer incubated at 16°C for 18 h. Transformation was performed using 50 µl *E. coli* competent cells (DH5-α) containing 10 µl of ligation product that was spread on Luria Bertani (LB) agar plates amended with kanamycin (50 µg/ml; Chem-Impex Int'l Inc., Wood Dale IL). To confirm the presence of the insert in the correct orientation, colony PCR was performed, the same colony was grown in liquid LB medium amended with kanamycin (50 µg/ml) for 18 h, and the plasmid construct was extracted using ZR plasmid miniprep (Zymo Research Corp) and sequenced (Eurofins Genomics, Louisville, KY).

***Agrobacterium tumefaciens*-mediated transient expression in *N. benthamiana* and *P. cinnamomi* inoculations.** Plasmid construct for WRKY51 (pXSN:WRKY51:HA) was electroporated into *A. tumefaciens* strain GV3101. GFP:HA construct was kindly provided by Dr. Hong-Gu Kang (pBIN). Positive transformants were grown on LB agar plates amended with kanamycin (50 µg/ml) for 48 h at 28°C. Individual bacterial colonies were grown in liquid LB medium (kanamycin, 50 µg/ml) for 18 h at 28°C. The suspensions were centrifuged at 3,000 g for 10 min at room temperature (Sorval ST 8R Centrifuge; Thermo Fisher Scientific, Waltham, MA), then resuspended in 3 ml of sterile infiltration buffer (10 mM $MgCl_2$, and 10mM MES, pH 5.5), and centrifuged at 3,000 g for 10 min. Pellets were resuspended in infiltration buffer with 200 µM acetosyringone

added to make agrobacteria suspension at OD_{600} = 0.7, and then incubated at room temperature for 2 h. Bacterial suspension carrying WRKY51 or GFP constructs were then infiltrated into 5-week-old *N. benthamiana* plants using a needleless 1 mL syringe to transiently express the proteins. The leaves containing the transiently expressed construct WRKY51 or GFP (control) were detached 3 hours post-infiltration and inoculated with zoospore suspension aliquots (1×10^4 spores/ml) of *P. cinnamomi* isolate 2113 as described above and incubated in a plant growth incubator at 25°C on inverted 15-cm 1.5% water agar plates for 4 days to evaluate the phenotype.

Western blot confirmation of transient expression in *N. benthamiana*. Two leaf discs (9 mm in diameter) were cut using a #4 cork borer from each leaf and frozen in liquid nitrogen immediately. Homogenized samples were combined with 60 µl 4× SDS buffer (200 mM Tris-HCl, pH 6.8, 400 mM dithiothreitol (DTT), 8% SDS, 0.4% bromophenol blue, and 40% glycerol) and incubated for 5 min at 95°C. Samples were centrifuged for 2 minutes at 16,200 g and 10 µl of supernatant was loaded into a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel. Gel was run at 60 volts for 2 h then put in transfer buffer (25 mM Tris, 190 mM glycine, and 20% methanol) for 10 min. Nitrocellulose membrane was blocked in 5% nonfat milk in 1×phosphate buffered saline with Tween-20 (PBST; 8mM Na_2HPO_4, 150mM NaCl, 2mM KH_2PO_4, 3mM KCl, and 0.05% Tween 20, pH 7.4) for 1 h, then incubated with a horseradish peroxidase (HRP)-conjugated anti-HA antibody (Roche Laboratories, Switzerland) at .0125 unit/ml for 18 h. Membrane was washed with 1× PBST 3 times for 10 min, then washed once in 1× phosphate buffered saline (PBS; 1.37 mM NaCl, 2.7

mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4) buffer. Membrane was transferred and visualized using a Bio Rad Chemi Doc MP (Bio-Rad Laboratories).

Defense response in *P. cinnamomi* inoculated avocado leaves. A differentially expressed defense response gene, Cytochrome P450 (Niben101Scf00158g04018.1), in both *N. benthamiana* and avocado (Mahomed and Van den Berg 2011; Reeksting et al. 2016) was chosen to evaluate the correlation between *N. benthamiana* model system and the avocado-*P. cinnamomi* system. Newly expanded leaves of Dusa® and PS.54 rootstocks were inoculated using the zoospore suspension of *P. cinnamomi* isolate 2113 as described above. The diameter of lesions on leaves were measured, and leaf discs were sampled at 24 hpi. The total RNA was extracted and RT-qPCR amplification of the target gene cytochrome P450 was conducted under the conditions described above, except that the primers of avocado cytochrome P450 (Bozak et al. 1990) and actin (Mahomed and Van den Berg 2011) were used (Table 4.1). Gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ (Ct = cycle threshold) comparative method. Eukaryotic actin was used as an endogenous reference gene for avocado. The results were reported as the mean ± standard error of three biological replications.

Statistical analysis of disease lesions. For comparisons of the disease lesion area on *N. benthamiana* or avocado leaves, variances were analyzed using Bartlett's test of homogeneity among repeated experiments, and homogenous data sets were combined. Mean lesion areas for each treatment were compared using analysis of variance (ANOVA). The differences between means of treatments were determined using Tukey's Honest Significant Difference (HSD). All statistical analyses were performed in R (3.1.3)

(R Core Team 2015) using the agricolae package (Mendiburu 2015). Differences were considered significant if $P \leq 0.05$.

RESULTS

RNAseq analysis of the inoculated and mock-infected *N. benthamiana*. The average total million reads for the sequenced mock-inoculated *N. benthamiana* samples were 66, 75, 75, 81, and 70 million for the 6, 12, 24, 36, and 48 hpi time points respectively. The average total reads for the inoculated *N. benthamiana* samples were 145, 123, 80, 62, and 70 million reads for the 6, 12, 24, 36, and 48 hpi time points respectively. The average percentage of reads mapped for the five-mock inoculated time points was between 86 and 88%. The first three inoculated time points, 6, 12, and 24 hpi, had an average percentage of mapped reads between 85 and 88%. The last two time points, 36 and 48 hpi, where microscopic and macroscopic cell death was observed only had 52 and 64% of their reads mapped respectively. The complete results for total reads, percentage of reads mapped, and percentage of uniquely mapped reads are listed in Table 4.2.

Transcriptional profile analysis of differentially expressed genes (DEGs) between the mock and in inoculated *N. benthamiana*. The total number of annotated proteins as well as DEGs are listed in Table 4.3. There were the highest number of both up-regulated and down-regulated DEGs at 24 hpi at 14,195 and 13,237 respectively. Using a cluster analysis of differentially expressed genes (Fig. 4.1), the function of

unknown genes can be recognized. In the hierarchical clustering, different areas with different colors, represent different groups of the cluster of genes up and down-regulated. This analysis shows two major clusters by treatment, one composed of all the mock uninoculated samples and inoculated samples corresponding to early time points (6 and 12 hpi). The other major cluster was composed of inoculated samples at 24, 36, and 48 hpi. These results indicate that the most drastic and significant changes in the host transcriptome occurs at the stages of the haustoria formation (24-36 hpi), transition from biotrophic to necrotrophic stage (36-48 hpi), and the visualization of cell death (48 hpi).

Using a Venn diagram differentially expressed genes (up-regulated and down-regulated) that are commonly and uniquely expressed between 6, 12, 24, 36, and 48 hpi can be identified (Fig. 4.2). Specifically, 64 DEGs were uniquely expressed at 6 hpi, 319 at 12 hpi, 5436 at 24 hpi, 2845 at 36 hpi, and 1128 at 48 hpi. DEGs associated with hormone signaling, transcription factors, *pathogenicity-related* (*PR*) genes, and *Receptor-like kinases* (*RLKs*) genes were identified (Fig. 4.3). At 6 hpi F-box proteins from the Jasmonic acid pathway were up-regulated 2.3-fold with a 1.4-fold down-regulation of an ethylene protinase inhibitor. A pathogenesis-related gene transcription activator was also up-regulated 4.4-fold and a cysteine rich receptor like protein kinase was up-regulated 3.8-fold. Members of the cytochrome P450 gene family were up-regulated ranging from 0.7- 6.1-fold with an average up-regulation of 3-fold (Supplementary Table 4.1).

At 12 hpi the only significantly up-regulated gene related to hormone signaling is *phenylalanine ammonia-lyase (PAL*) which was up-regulated 2.8-fold. Multiple transcription factors were differentially expressed. Genes encoding ethylene associated,

WRKY, and pathogenesis related transcription factors were up-regulated 8-, 12.2-, and 6-fold respectively. *Pathogenesis-related protein 1 (PR-1*) and *peroxidase* (*PR-9*) genes were also up-regulated 5.6- and 10.6-fold. *LRR receptor-like kinase* and *cysteine rich receptor like protein kinase* genes were both up-regulated 5- and 11.5-fold respectively. Finally, several members of the *cytochrome P450* gene family were up-regulated ranging from 1.5- 7.9-fold with an average up-regulation of 3.9-fold (Supplementary Table 4.1). A gene encoding a salicylic acid binding protein (Niben101Scf02195g-04001.1) in the SA pathway was significantly down-regulated at 24- and 36-hpi with a 5- and 10.5-fold down-regulation respectively. A number of other differentially expressed genes were at least 10-fold up-regulated at 24 hpi including: *WRKY51* (ranging from 9.5- 13.5-fold with an average up-regulation of 11.2-fold) (Supplementary Table 4.2)*, PR-1, PR-4, PR-9*, and the cysteine rich receptor like protein kinase. At 36 hpi; ethylene response, *WRKY51* (ranging from 11.2- 12.7-fold with an average up-regulation of 12.2-fold) (Supplementary Table 4.2), pathogenesis-related gene transcription activator, heat stress, *PR-1, PR4, PR9*, and cytochrome P450 (13.3-fold) (Supplementary Table 4.1) were all up-regulated at least 10-fold. Genes up-regulated more than ten-fold at 48 hpi include: *WRKY51*, (10.8-fold) (Supplementary Table 4.2) and heat stress associated genes, *PR-1*, and *PR-4* (Fig. 4.3).

Functional categorization of DEGs by Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway. In order to further clarify molecular and biological functions of the genes, these DEGs were mapped to the KEGG database. *Metabolism and energy.* A number of metabolic and energy pathways are down-regulated at 6 hpi and up-

regulated at 12 hpi. At 6 hpi photosynthesis, ether lipid metabolism, and linoleic acid metabolism have enriched genes that are down-regulated with adjusted p-values of 0.005, 0.01, and 0.03 respectively. (Fig. 4.4). At 12 hpi metabolic and energy pathways including: carbon metabolism, endocytosis, glycolysis / gluconeogenesis, and citrate cycle have enriched genes that are up-regulated with adjusted p-values of 0.001, 0.0005, 0.02, and 0.00005 respectively.

At 24 hpi, two KEGG pathways both carbon fixation (photosynthesis) and glyoxylate / dicarboxylate (biosynthesis of carbohydrates from fatty acids or two-carbon precursors) (metabolism) have enriched genes that are significantly down-regulated with adjusted p-values of 0.014 and 0.018 (Fig. 4.4). At 36 hpi endocytosis had 140 genes up-regulated (adjusted p-value =0.002) and carbon metabolism, photosynthesis, carbon fixation, and glyoxylate / dicarboxylate metabolism all have enriched genes that are down-regulated with adjusted p-values of 0.005, 0.003, 0.0003, and 0.006 respectively (Fig 4.4). At 48 hpi up-regulated enriched genes included oxidative phosphorylation and glycolysis / gluconeogenesis (adjusted p-value 0.0006 and 0.02). However, a large number of down-regulated metabolic and energy producing gene groups were enriched including: carbon metabolism, carbon fixation, glyoxylate / dicarboxylate metabolism, glycene, serine, and threonine metabolism, porphyrin and chlorophyll metabolism, and circadian rhythm with adjusted p-values of 0.01, 0.00007, 0.0002, 0.01, 0.002 and 0.02 respectively.

Biosynthesis. Up-regulated enriched gene groups associated with biosynthesis were strongly represented at 6- and 12-hpi (Fig. 4.4). The group with the highest number

of genes at both 6- and 12-hpi was secondary metabolites (adjusted p-value 0.00009 and 0.04). Pathways known to be involved in plant defense response against a wide range of pathogens and pests such as the sesquiterpenoid and triterpenoid biosynthesis, flavonoid biosynthesis, and the phenylpropanoid pathways (SA biosynthesis, lignin, and antimicrobial compounds) were among the most significantly enriched pathways up-regulated at these time points. A high production of secondary metabolites is often associated with plant defense. Terpenoid and amino acid biosynthesis were also highly represented, both of which have important roles in plant defense. The only down-regulated enriched gene groups in biosynthesis were carotenoids, and valine, leucine, and isoleucine biosynthesis at 48-hpi (adjusted p-value 0.01 and 0.03).

*Plant-pathogen interactions***.** Up-regulated enriched genes associated with plant-pathogen interactions are highly represented at all the time points except 24-hpi (Fig. 4.4). At 6 hpi, pattern recognition receptors (PRRs) such us *Flagellin Sensing2 receptor* (*FLS2*) and *xylanase/ETHYLENE INDUCING XYLANASE2 EIX2* receptor (*Eix2*) (fungal resistance) were found significantly enriched. Genes encoding for calcium binding (Niben101Scf05029g02016.1) and heat shock proteins (Niben101Scf04847g-03018.1) were also enriched at 12 hpi.

Interestingly, at 36-hpi, genes encoding calcium-dependent protein kinases (CDPKs), MAPKK, WRKY transcription factors, PRRs (EIX2 and the chitin elicitor receptor kinase 1), PR-1, several R proteins, and EDS1 (essential component of *R* gene-mediated disease resistance) were significantly enriched. In addition, at this time point up-regulated genes associated with the phagosome were numerous (adjusted *P*-value

0.04). Phagosome formation is crucial for host defense against pathogens. There were also a large number of down-regulated enriched genes associated with plant hormone signal transduction at both 12 and 48-hpi (adjusted *P*-value 0.00000002 and 0.05). Genes involved in plant hormone signaling transduction pathway such as *auxin-induced protein 22D* (Niben101Scf07160g00025.1) and *small auxin-up protein 58* (Niben101Scf08141g-01009.1) were significantly down-regulated at 12-hpi. At 48 hpi, genes encoding an auxin-binding protein (Niben101Scf02749g04008.1) and an auxin responsive protein (Niben101Scf-02635g00003.1) were significantly down-regulated (Fig. 4.4).

Transcription, translation, and replication. There were a large number of up-regulated enriched genes associated with the ribosome and protein processing in the endoplasmic reticulum at 48-hpi, 321 and 189 respectively (adjusted *P* value 0.0000000009 and 0.0007) (Fig. 4.4). There were also 72 enriched genes associated with the ribosome down-regulated at 12-hpi (adjusted *P* value 0.00000000003). Down-regulated genes associated with the spliceosome were enriched at 12 and 36-hpi (adjusted *P* value 0.006 and 0.008).

Similarities with previous RNAseq studies in avocado and other tree hosts. There were many common differentially expressed plant defense genes that were up-regulated in *P. cinnamomi* infected *N. benthamiana* leaves when compared to root-inoculated avocado, chestnut, and eucalyptus (Fig. 4.5). Specifically, *PAL*, *Thaumatin*, *Allene oxide synthase*, *F-box proteins*, and *cytochrome P450* were also significantly up-regulated in avocado and *L. longifolia* roots. Genes encoding several members of the WRKY transcription factors were up-regulated in avocado, eucalyptus, chestnut, and *L.*

longifolia roots. *Glutathione S-transferase* gene was up-regulated significantly in avocado and *L. longifolia* roots. These and other defense related genes commonly expressed in avocado, other model systems, and the *N. benthamiana* pathosystem support the use of *N. benthamiana* to investigate defense gene response to *P. cinnamomi*.

qPCR validation of RNAseq results. The qPCR assays showed up-regulation of all six genes defense related genes tested (Fig. 4.6), which was similar to the RNAseq expression results. The genes used to validate the RNAseq were *aristolochene synthase* (Niben101Scf00700g00005.1) (EAS), *cytochrome P450* (Niben101Scf00158g04018.1) (CYP), *glutathione S- transferase* (Niben101Scf03147g10015.1) (GST), *phenylalanine ammonia-lyase* (Niben101Scf05617g00005.1) (PAL), *premnaspirodiene oxygenase* (Niben101Ctg13347g00002.1) (HPO), and *WRKY transcription factor 51* (Niben101Scf01942g04001.1) (WRKY51). The $\log_2$ fold change values obtained by RNAseq showed a strong correlation ($r = 0.91$, $P = 0.01$) with the values obtained by qPCR based on the Pearson's correlation test. This finding suggests that sequencing provided reliable results, demonstrating the reproducibility and accuracy of the technique.

Functional assays of WRKY51 in *N. benthamiana*. *N. benthamiana* detached leaves infiltrated with WRKY51 over-expression construct showed a significant decrease in lesion formation compared to the GFP control at 48, 72, and 96 hpi (Fig. 4.7). At 48-hpi a necrotic lesion had begun to develop in all of the GFP control leaves, compared to just one out of the six WRKY51 inoculated leaves. At 72-hpi all of the necrotic lesions on the GFP control leaves had expanded compared to just two leaves in the WRKY51 group with one of the lesions just beginning to develop at 72-hpi. The final measurements

at 96 hpi showed normal lesion development in all six of the GFP control leaves inoculated and still just two leaves in the experimental group with lesions. A western blot confirmed the expression of the transiently over-expressed WRKY51 protein at the expected molecular weight of 28.0 kilodaltons (Fig. 4.8). This experiment was repeated at least once.

Defense response in *P. cinnamomi* inoculated avocado leaves. An evaluation of the correlation of the *N. benthamiana* model system and avocado-*P. cinnamomi* pathosystem was made on tolerant Dusa® and susceptible PS.54 rootstocks leaves. The lesion area on detached leaves of the susceptible PS.54 was significantly larger compared to the tolerant Dusa® (Fig. 4.9). The expression levels of plant defense gene *cytochrome P450* in inoculated avocado leaves was compared to that in *N. benthamiana* model system, as well as between the tolerant and susceptible avocado rootstocks. The qPCR results show that CYP was up-regulated in both avocado-*P. cinnamomi* pathosystem and the *N. benthamiana* model system. A comparison of CYP relative expression levels between Dusa® and PS.54 showed significantly higher expression of this plant defense gene in the susceptible rootstock (Fig. 4.10).

DISCUSSION

In this study we have elucidated the gene expression in response to *P. cinnamomi* infection in the *N. benthamiana* model system. By analyzing the transcriptome of *N. benthamiana* with RNAseq at five critical time points during the infection it was possible

to identify important defense pathways using our model system. As early as 6-hpi there is a response by the infected host. We see a significant number of genes involved in the biosynthesis of secondary metabolites up-regulated. Specifically, known defense-related biosynthesis pathways such as flavonoid, terpenoid, and the phenilpropanoid pathways were enriched. Numerous plant-pathogen interaction genes were also enriched indicating the initiation of an active defense response to the pathogen. The highest number of enriched genes were involved in the biosynthesis of secondary metabolites at both 6 and 12-hpi. There was also a significant down-regulation of genes associated with the ribosome and plant hormone signal transduction especially auxin related genes. There is also a down-regulation in photosynthesis and metabolic pathways indicative of a decrease in resources allocated to growth and energy production in response to pathogen detection. The DEGs analysis confirms this early defense response with up-regulated hormone signaling, transcription factors, pathogen related genes, and resistance genes. At 24 hpi the KEGG enriched terms show a decrease in carbon fixation and metabolism which supports the allocation of resources towards plant defense. Salicylic acid binding is also down-regulated 5-fold which could be the result of pathogen effectors subverting the defense response. At the same time, we found transcription factors, PR genes, R genes, and anti-fungal genes that were up-regulated over 10-fold. Although *P. cinnamomi* is attempting to subvert the host defenses, the extensive colonization and intracellular growth (Belisle et al. 2018) at this stage of the infection has induced an extreme response in *N. benthamiana*. At 36 hpi we found that besides significant up regulation in the plant-pathogen interaction KEGG enrichment pathway there was up regulation of genes

involved in endocytosis and phagosomes. Interestingly this is the time point where plant cell death becomes apparent in previously stained images of the infection process (Belisle et al. 2018) and is thought to be the stage where *P. cinnamomi* becomes necrotrophic in its infection strategy. There is also a 10-fold down regulation of SA binding and 5 to 10-fold up regulation of the JA pathway that further supports the necrotrophic infection occurring at this stage. At 48 hpi the KEGG pathway shows that 321 unigenes are involved in the up regulation of ribosome function. At the same time 770 unigenes are involved in the down regulation of metabolic pathways. The expanding necrotic lesion visible at this stage supports the gene expression pattern. Numerous defense genes are also being highly expressed at this time point including the continued up-regulation of the JA pathway.

PAMP-triggered immunity (PTI) is the plants first layer of defense against plant pathogens. Plants have developed pattern recognition receptors (PRR) that initiate a defense response before the pathogen is able to infect the plant. This early PTI response is at a time in our model system when many of the encysted zoospores on the inoculated leaf surface haven't germinated and there is no intracellular penetration (Belisle et al. 2018). PTI response is linked to reactive oxygen species (ROS) production, lignin and callose reinforcement, and the up-regulation of *pathogenesis-related* (PR) genes. Genes encoding Glutathione S-transferase, which serves to protect plant cells from ROS production was significantly up-regulated in our system as well as previously identified as being up-regulated during infection in *Arabidopsis*, *Z. mays*, and avocado (Allardyce et al. 2013; Reeksting et al. 2014; Rookes et al. 2008). PR-genes up-regulated

significantly in *N. benthamiana* in response to *P. cinnamomi* infection included; *PR-1*, *PR-4* (chitin binding), *PR-5* (thaumatin-like protein), and *PR-9* (peroxidase). *PR-5* was also up-regulated in infected avocado roots and *PR-1* and *PR-5* were up-regulated in eucalyptus roots (Mahomed and Van den Berg 2011; Meyer et al. 2016).

Hormone signaling plays an important role in a plants response to various pathogens and the pathway initiated varies depending on the type of pathogen. The jasmonic acid and ethylene signaling pathways are normally initiated in response to necrotrophic pathogens. The cytochrome P450 (CYP) superfamily is the largest enzymatic protein family in plants (Xu et al. 2015). CYP genes are involved in hormone signaling and associated with the JA pathway (Aubert et al. 2015). CYP has been described as a JA-responsive gene which was up-regulated 37.75-fold in *L. longifolia* (Islam et al. 2017). Numerous genes in this family were up-regulated in all of the five time points we analyzed. Up-regulation of CYP has also been found in inoculated avocado (Mahomed and Van den Berg 2011). Some interesting and less well described anti-fungal genes in the CYP family, premnaspirodiene oxygenase (19.3-fold at 24 hpi) and aristolochene synthase (17-fold at 24 hpi) were identified that would be good candidates for further analysis. F-box proteins associated with the JA pathway were up-regulated in 4 out of 5 of the timepoints in our system. The up-regulation of this gene in response to *P. cinnamomi* infection has also been discovered in avocado as well as numerous other model systems indicating a similar response among these different plants. Allene oxide synthase genes involved in JA biosynthesis (Allardyce et al. 2013), were also up-regulated in our system. JA response is traditionally associated with necrotrophic

pathogen defense, but recent studies have shown that biotrophic pathogens such as *Plasmopara viticola* (Guerreiro et al. 2016) and hemi-biotrophs like *P. infestans* (Kamoun et al. 2015) and *P. cinnamomi* (Reeksting et al. 2014) can also trigger the activation of a JA triggered response.

There were also some differentially expressed genes that are normally associated with the SA pathway discovered in the *N. benthamiana* system including up-regulated *PAL* genes and down-regulated SA binding genes. PAL is the key enzyme for the phenylpropanoid pathway which is involved in SA biosynthesis, lignin, and antimicrobial compounds such as flavonoids and phytoalexins (Islam et al. 2017). Auxin signaling has been shown to play and important role in the induction of resistance to *P. cinnamomi* (Eshraghi et al. 2014). Plants using more than one defense pathway in response to *P. cinnamomi* infection has been seen previously in avocado and *L. longifolia* (Islam et al. 2017; Reeksting et al. 2014) but was not found in *Z. mays* (Allardyce et al. 2013). Interestingly, genes encoding WRKY transcription factors were significantly up-regulated at all of the timepoints in our system. In plants, WRKY transcription factors are encoded by a large family of genes, and they are involved in abiotic and biotic stress and are activated by pathogen perception (Xu et al. 2015). *WRKY* genes have been previously shown to be direct transcriptional targets of NPR1 in response to SA abundance (Wang et al. 2006). The significant up-regulation of WRKY transcription factors in our model system may indicate that there is some JA/ SA crosstalk in the infected samples.

Up regulation of WRKY transcription factors in response to *P. cinnamomi* infection was also found in eucalyptus, chestnut, and *L. longifolia* roots. (Islam et al.

2017; Meyer et al. 2016; Santos et al. 2017). WRKY51 transcription factor was selected as an ideal candidate for functional experiments because of its consistent up-regulated expression from 12 to 48 hpi shown in the RNAseq data and the uniform results during the qPCR validation. For the many reasons listed above WRKY51 transcription factor was chosen to functionally validate in our *N. benthamiana* model system. The transient over-expression of the WRKY51 construct was carefully timed through numerous experiments to discover at what point, if any, in comparison with the *P. cinnamomi* infection would the expression of this transcription factor have the greatest effect on the colonization of the pathogen. Transient expression of the WRKY51 protein 3 hours before inoculation with *P. cinnamomi* zoospores produced the clearest phenotype difference between the experimental and control groups. This early transient expression may induce an early defense response before the pathogen has a chance to colonize the host. The RNAseq data in this study and others, and the functional assay using WRKY51 confirm that a close analysis of its expression in avocado is warranted.

The RNAseq data as well as the functional work in this study provide a wealth of information concerning host defense response to *P. cinnamomi* infection. It is important however, to begin to make connections back to avocado which is the economically important crop we are interested in understanding more completely. By using the data obtained in this study and developing functional experiments that overcome the limitations associated with tree crops such as avocado we will be able to better address the long-term breeding concerns of avocado growers. Genes identified in our model system such as *cytochrome P450* (CYPs) or identified and validated such as *WRKY51* can

be used for marker assisted breeding. The avocado qPCR data for *cytochrome P450* is an important first step in the goal to learn more about avocado defense gene response directly. The expression of cytochrome P450 in inoculated detached avocado leaves was similar to the expression in detached *N. benthamiana* leaves in both PS.54 (susceptible) and Dusa® (tolerant) rootstocks. Since cytochrome P450 has already been identified to be significantly up-regulated in *P. cinnamomi* infected avocado roots (Mahomed and Van den Berg 2011), it is reasonable to infer that this defense gene is similarly expressed in both roots and shoots. These universally expressed plant defense genes will provide vital information for resistance breeding projects in avocado. The next step is to find a functionally validated defense gene in our model system that is differentially expressed in the susceptible and tolerant avocado varieties (leaf and roots) and also transiently overexpress the avocado candidate genes homologs in *N. benthamiana* for functional validation. This would be an ideal candidate for marker assisted breeding. Using our model system, it is possible to identify such a candidate.

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REFERENCES

1. Aubert, Y., Widemann, E., Miesch, L., Pinot, F., and Heitz, T. 2015. CYP94-mediated jasmonoyl-isoleucine hormone oxidation shapes jasmonate profiles and attenuates defence responses to *Botrytis cinerea* infection. J. Exp. Bot. 66:3879-3892.

2. Allardyce, J. A., Rookes, J. E., Hussain, H. I., and Cahill, D. M. 2013. Transcriptional profiling of *Zea mays* roots reveals roles for jasmonic acid and terpenoids in resistance against *Phytophthora cinnamomi*. Funct. Integr. Genomics 13:217-228.

3. Belisle, R., McKee, B., Hao, W., Crowley, M., Arpaia, M. L., Miles, T. D., Dr. James E. Adaskaveg, J. E., and Manosalva, P. 2018. Phenotypic characterization of genetically distinct *Phytophthora cinnamomi* isolates from avocado. Phytopathology 10.1094/PHYTO-09-17-0326-R.

4. Bender, G. S., Menge, J. A., and Arpaia, M. L. 2004. Avocado rootstocks. Pages 45-53 in: Avocado Production in California: A Cultural Handbook for Growers. Book 1: Background Information, 2nd ed. G. S. Bender ed. University of California Cooperative Extension, San Diego County and The California Avocado Society, Oakland, CA.

5. Benjamini, Y. and Hochberg, Y. 1995. Controlling the false discovery rate: a practical and powerful approach to multiple testing. J. R. Stat. Soc. Series B 85:289-300.

6. Boesewinkel, H. J. 1976. Storage of fungal cultures in water. Trans. Br. Mycol. Soc. 66:183-185.

7. Bombarely, A., Rosli, H., Verbalov, J., Moffett, P., Mueller, L. A., and Martin, G. B. 2012. A draft genome sequence of *Nicotiana benthamiana* to enhance molecular plant-microbe biology research. Mol. Plant Microbe Interact. 25:1523-1530.

8. Bozak, K. R., Yu, H., Sirevag, R. and Christoffersen, R. E. 1990. Sequence analysis of ripening-related cytochrome P-450 cDNAs from avocado fruit. Proc. Natl. Acad. Sci. U.S.A. 87:3904-3908.

9. Chen, S., Songkumarn, P., Liu, J., and Wang, W. 2009. A versatile zero background T-vector system for gene cloning and functional genomics. Plant Physio. 150:1111-1121.

10. Chen, X., Xing, Y., Li, Y., Tomg, Y., and Xu, J. 2013. RNA-Seq reveals infection-related gene expression changes in *Phytophthora capsici*. PLOS One 8:e74588.

11. Dalio, R. J., Maximo, H. J., Oliveira, T. S., Dias, R. O., Breton, M. C., Felizatti, H., and Machado, M. 2018. *Phytophthora parasitica* effector PpRxLR2 suppresses *Nicotiana benthamiana* immunity. Mol. Plant Microbe Interact. 31:481-493.

12. Denman, S., Kirk, S. A., Brasier, C. M., and Webber, J. F. 2005. *In vitro* leaf inoculations studies as an indication of tree foliage susceptibility to *Phytophthora ramorum* in the UK. Plant Pathol. 54:512-521.

13. Dong, L., Cheng, Y., Wu, J., Cheng, Q., Li, W., Fan, S., Jiang, L., Xu, Z., Kong, F., Zhang, D., Xu, P., and Zhang, S. 2015. Overexpression of GmERF5, a new member of the soybean EAR motif-containing ERF transcription factor, enhances resistance to *Phytophthora sojae* in soybean. J. Exp. Bot. 66:1-13.

14. Ekchaweng, K., Evangelisti, E., Schornack, S., Tian, M., and Churngchow, N. 2017. The plant defense and pathogen counterdefense mediated by *Hevea brasilensis* serine protease *HbSPA* and *Phytophthora palmivora* extracellular protease inhibitor *PpEPI10*. PLOS One 12:e0175795.

15. Erwin, D. C., and Ribeiro, O. K. 1996. Phytophthora Diseases Worldwide. APS Press, The American Phytopathological Society, St. Paul, MN.

16. Esharghi, L., Anderson, J., Aryamanesh, N., Shearer, B., McComb, J., Hardy, G. E. St. J., and O'Brien, P. A. 2011. Phosphite primed defense responses and enhanced expression of defense genes in *Arabidopsis thaliana* infected with *Phytophthora cinnamomi*. Plant Pathol. 60:1086-1095.

17. Eshraghi, L., Anderson, J. P., Aryamanesh, N., McComb, J. A., Shearer, B., and Hardy, G. E. St. J. 2014. Suppression of the auxin response pathway enhances susceptibility to *Phytophthora cinnamomi* while phosphite-mediated resistance stimulates the auxin signalling pathway. BMC Plant Biol. 14:68.

18. Evangelisti, E., Gogleva, A., Hainaux, T., Doumane, M., Tulin, F., Quan, C., and Schornack, S. 2017. Time-resolved dual transcriptomics reveal early induced *Nicotiana benthamiana* root genes and conserved infection-promoting *Phytophthora palmivora* effectors. BMC Biol. 15:39.

19. Goodin, M. M., Zaitlin, D., Naidu, R. A., and Lommel, S. A. 2008. *Nicotiana benthamiana*: its history and future as a model for plant-pathogen interactions. Mol. Plant Microbe Interact. 21:1015-1026.

20. Guerreiro, A., Figueiredo, J., Sousa Silva, M., and Figueiredo, A. 2016. Linking Jasmonic acid to grapevine resistance against the biotrophic oomycete *Plasmopara viticola*. Front. Plant Sci. 7:565.

21. Gunning, T. K., Conlan, X. A., Parker, R. M., Dyson, G. A., Adams, M. J., Barnett, N. W., and Cahill, D. M. 2013. Profiling of secondary metabolites in blue lupin inoculated with *Phytophthora cinnamomi* following phosphite treatment. Funct. Plant Biol. 40:1089-1097.

22. Hardham, A. R. 2005. *Phytophthora cinnamomi*. Mol. Plant Pathol. 6:589-604.

23. Hardham, A. R., and Blackman, L. M. 2018. *Phytophthora cinnamomi*. Mol. Plant Pathol. 19:260-285.

24. Helliwell, E. E., Vega-Arreguin, J., Shi, Z., Bailey, B., Xiao, S., Maximova, S. N., Tyler, B. M., and Guiltinan, M. J. 2016. Enhanced resistance in *Theobroma cacao* against oomycete and fungal pathogens by secretion of phosphatidylinositol-3-phosphate-binding proteins. Plant Biotechnol. J. 14:875-886.

25. Huisman, R., Bouwmeester, K., Brattinga, M., Govers, F., Bisseling, T., and Limpens, E. 2015. Haustorium formation in *Medicago truncatula* roots infected by *Phytophthora palmivora* does not involve the common endosymbiotic program shared by arbuscular mycorrhizal fungi and rhizobia. Mol. Plant Microbe Interact. 28:1271-1280.

26. Islam, M.T., Hussain, H. I., Rookes, J.E., and Cahill, D.M. 2017. Transcriptome analysis, using RNA-Seq of *Lomandra longifolia* roots infected with *Phytophthora cinnamomi* reveals the complexity of the resistance response. Plant Biol. 20:130-142.

27. Kamoun, S., Furzer, O., Jones, J. D. G., Judelson, H. S., Ali, G. S., Dalio, R. J., Roy, S. G., Schena, L., Zambounis, A., Panabières, F., Cahill, D., Ruocco, M., Figueiredo, A., Chen, X. R., Hulvey, J., Stam, R., Lamour, K., Gijzen, M., Tyler, B. M., Grünwald, N. J., Mukhtar, M. S., Tomé, D. F., Tör, M., Van Den Ackerveken, G. , McDowell J., Daayf, F., Fry, W. E., Lindqvist-Kreuze. H., Meijer, H. J., Petre, B., Ristaino, J., Yoshida, K., Birch, P. R., and Govers, F. 2015. The top 10 oomycete pathogens in molecular plant pathology. Mol. Plant Pathol. 16:413-434.

28. Kim, D., Pertea, G., Trapnell, C., Pimentel, H., Kelley, R., and Salzberg, S. L. 2013. TopHat2: accurate alignment of transcriptomes in the presence of insertions, deletions and gene fusions. Genome Biol. 14:R36.

29. Londsdale, J. H., Botha, T., and Kotze, J. M. 1988. Preliminary trials to assess the resistance of three clonal avocado rootstocks to crown canker caused by *Phytophthora cinnamomi*. South African Avocado Growers Association Yearbook 11:35-37.

30. Mahomed, W., and Van den Berg, N. 2011. EST sequencing and gene expression profiling of defence-related genes from *Persea americana* infected with *Phytophthora cinnamomi*. BMC Plant Biol. 11:167.

31. Matkin, O. A., and Chandler, P. A. 1957. The U.C. type soil mixes. Pages 68-85 in: The U.C. System for Producing Healthy Container-Grown Plants. K. F. Baker, ed. University of California division of Agriculture and Natural Resources, Manual 23, Berkeley, CA.

32. Manosalva, P., Manohar M., von Reuss, S. H., Chen, S., Koch, A., Kaplan, F., Choe, A., Micikas, R. J., Wang, X., Kogel, K. H., Sternberg, P. W., Williamson, V. M., Schroeder, F. C., and Klessig, D. F. 2015. Conserved nematode signaling molecules elicit plant defenses and pathogen resistance. Nat. Commun. 6:7705.

33. Mendiburu, F. de. 2015. agricolae: Statistical Procedures for Agricultural Research. R Package Version 1.2-3. http://CRAN.R-project.org/package=agricolae.

34. Meyer, F. E., Shuey, L. S., Ramsuchit, S., Mamni, T., Berger, D. K., Van den berg, N., Myburg, A. A., and Naidoo, S. 2016. Dual RNA-sequencing of *Eucalyptus nitens* during *Phytophthora cinnamomi* challenge reveals pathogen and host factors influencing compatibility. Front. Plant Sci. 7:191.

35. R Core Team. 2015. R: A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing. http://www.R-project.org.

36. Reeksting, B. J., Coetzer, N., Mahomed, W., Engelbrecht, J., and van den Berg, N. 2014. De novo sequencing, assembly, and analysis of the root transcriptome of *Persea americana* (Mill.) in response to *Phytophthora cinnamomi* and flooding. PLoS ONE 9:e86399.

37. Reeksting, B. J., Coetzer, Olivier, N. A., and van den Berg, N. 2016. Transcriptome responses of an ungrafted Phytophthora root rot tolerant avocado

(*Persea americana*) rootstock to flooding and *Phytophthora cinnamomi*. BMC Plant Biol. 16:205.

38. Ribeiro, O. K. 1978. A Source Book of the Genus *Phytophthora*. J. Cramer, Vaduz, Liechtenstein.

39. Robin, C., and Guest, D. 1994. Characterization of pathogenicity of *Phytophthora parasitica* isolates by stem and detached-leaf inoculations in four tobacco cultivars. N. Z. J. Crop Hortic. Sci. 22:159-166.

40. Robinson, L. H., and Cahill, D. M. 2003. Ecotypic variation in the response of *Arabidopsis thaliana* to *Phytophthora cinnamomi*. Australa. Plant pathol. 32:53-64.

41. Rookes, J. E., Wright, M. L., and Cahill, D. M. 2008. Elucidation of defense responses and signaling pathways induced in *Arabidopsis thaliana* following challenge with *Phytophthora cinnamomi*. Physiol. Mol. Plant Pathol. 72:151-161.

42. Santos, C., Duarte, S., Tedesco, S., Fevereiro, P., and Costa, R. L. 2017. Expression profiling of castanea genes during resistant and susceptible interactions with the oomycete pathogen *Phytophthora cinnamomi* reveal possible mechanisms of immunity. Front. Plant Sci. 8:515.

43. Shands, A. C., Yamagata, J. S., Wright, A. F., and Miles, T. D. 2016. First report of *Phytophthora cinnamomi* causing root rot of Southern highbush blueberry in California. Plant Dis. 100:537.

44. Vega-Arreguin, J. C., Jalloh, A., Bos, J. I., and Moffett, P. 2014. Recognition of an Avr3a homologue plays a major role in mediating nonhost resistance to *Phytophthora capsici* in *Nicotiana* species. MPMI 27:770-780.

45. Xu, X., Wang, R., Chao, J., Lin, Y., Jin, Q., He, X., Luo, S., and Wu, T. 2015. The expression patterns of *Cucumis sativus* WRKY (CsWRKY) family under the condition of inoculation with *Phytophthora melonis* in disease resistant and susceptible cucumber cultivars. Can. J. Plant Sci. 95:1121-1131.

46. Wang, D., Amornsiripanitch, N., and Dong, X. 2006. A genomic approach to identify regulatory nodes in the transcriptional network of systemic acquired resistance in plants. PLoS Pathog. 2:e123.

47. Zentmyer, G. A. 1980. *Phytophthora cinnamomi* and the diseases it causes. Monograph No. 10. The American Phytopathological Society, St. Paul, MN.

Table 4.1. Quantitative PCR (qPCR) and plasmid construct oligonucleotides used in this study

Target gene	Primer ID	Sequences 5' to 3'	Origin
***Nicotianae benthamiana* qPCR primers**			
Aristolochene synthase (EAS)	00700_asynth_qF1	TCCGCAAATTCCAAGATGAA	This study
	00700_asynth_qR1	CGACTCTAGGAACGCCCTTG	This study
Cytochrome P450 (CYP)	11882_cytochrome _qF3	TGGAGGATGGCCGGTTATAG	This study
	11882_cytochrome _qR3	CGATTTTTCCGCCAGTAAGG	This study
Glutathione S-transferase (GST)	03147_glutathione _qF1	GGGGTCTGGGGAGGGTAATA	This study
	03147_glutathione _qR1	GCCTGGATGGAAATGGAGAG	This study
Phenylalanine ammonia-lyase (PAL)	qNbPAL2 F	GAGTGCTAGATGTGACTTGG	This study
	qNbPAL2 R	CTAGCAGAGTGGAAGAGGAG	This study
Premnaspirodiene oxygenase (HPO)	13347_prox_qF1	CATGCTGACATGGGATTTCG	This study
	13347_prox_qR1	AGCCCTGCCTACACTTGCAT	This study
WRKY transcription factor 51 (WRK51)	05057_WRKY51_ qF2	GTTCAAGTGGAGGTTGCAAGG	This study
	05057_WRKY51_ qR2	ACCTCGGCTTAATCGGCTCT	This study
Elongation factor 1-alpha 1 (EF1-α1)	NbEF1A_F1	AGCTTTACCTCCCAAGTCATC	This study
	NbEF1A_R1	AGAACGCCTGTCAATCTTGG	This study
Avocado qPCR primers			
Cytochrome P450	AvCytochrome qP450F2	TAGCCGACCATCCACAACTG	GenBank: M32885.1[z]
	AvCytochrome qP450R2	CTTCCCAAAAGCAACCCTTG	GenBank: M32885.1[z]
Actin	Av actin qF1	CGAAGATGGCTGATGCAGAG	This study
	Av actin qR1	TCTCCATGTCGTCCCAGTTG	This study
Plasmid construct primers			
WRKY transcription	05057_WRKY51_ cF2	TATGAATCAAGATCATGGCCA CAATATGTATCAAG	This study

factor 51 (WRKY51)	05057_WRKY51_ cR2	TCACCAGAAAGAATCTGCTTG CAAAGT	This study

[z] Bozak, K. R., Yu, H., Sirevag, R. and Christoffersen, R. E. 1990. Sequence analysis of ripening-related cytochrome P-450 cDNAs from avocado fruit. Proc. Natl. Acad. Sci. U.S.A. 87:3904-3908.

Table 4.2. Summary of RNAseq analysis of *Phytophthora cinnamomi* inoculated and mock *Nicotianae benthamiana* leaves at 6, 12, 24, 36, and 48 h post inoculation (hpi)

Time point	Sample type	Sample no.	Total reads	Total mapped	Multiple mapped	Uniquely mapped
6hpi	Mock	1	65425404	85.82%	5.28%	80.54%
		2	61477470	86.04%	5.22%	80.82%
		3	71427534	85.56%	5.40%	80.17%
	Inoculated	1	190782578	88.69%	5.53%	83.15%
		2	121629686	88.25%	5.31%	82.94%
		3	121510634	88.46%	5.35%	83.12%
12hpi	Mock	1	65758586	86.61%	5.25%	81.36%
		2	93810268	85.91%	5.17%	80.73%
		3	64736392	90.06%	5.99%	84.07%
	Inoculated	1	128759056	86.05%	5.11%	80.93%
		2	120171542	86.91%	5.02%	81.89%
		3	120137480	86.65%	5.22%	81.43%
24hpi	Mock	1	69009154	86.18%	4.94%	81.24%
		2	66248812	85.68%	5.07%	80.61%
		3	91127546	87.47%	5.09%	82.39%
	Inoculated	1	88193316	82.04%	4.19%	77.85%
		2	90914562	86.23%	4.58%	81.64%
		3	60740862	86.87%	4.35%	82.52%
36hpi	Mock	1	79516264	87.16%	5.11%	82.05%
		2	82941102	87.04%	5.10%	81.94%
		3	80439236	87.29%	5.05%	82.24%
	Inoculated	1	62235380	50.66%	2.70%	47.96%
		2	62242218	52.33%	2.75%	49.59%
48hpi	Mock	1	83058336	87.47%	4.82%	82.65%
		2	59326048	86.63%	4.62%	82.01%
		3	66848446	86.54%	4.74%	81.79%
	Inoculated	1	60306854	62.05%	2.81%	59.25%
		2	79909262	64.82%	2.83%	61.99%

Table 4.3. Summary of differently expressed genes between the *Phytophthora cinnamomi* inoculated and mock *Nicotiana benthamiana* leaves at 6, 12, 24, 36, and 48 h post inoculation (hpi)

	6hpi	**12hpi**	**24hpi**	**36hpi**	**48hpi**
Proteins	76,378	76,378	76,378	76,378	76,378
Proteins (mapped to genome)	65,718	65,718	65,718	65,718	65,718
Differentially expressed genes (DEGs)	1,293	6,599	27,432	23,360	17,583
Up-regulated	651	4,747	14,195	11,651	9,546
Down-regulated	642	1,852	13,237	11,709	8,037
DEGs%	2%	10%	42%	36%	27%

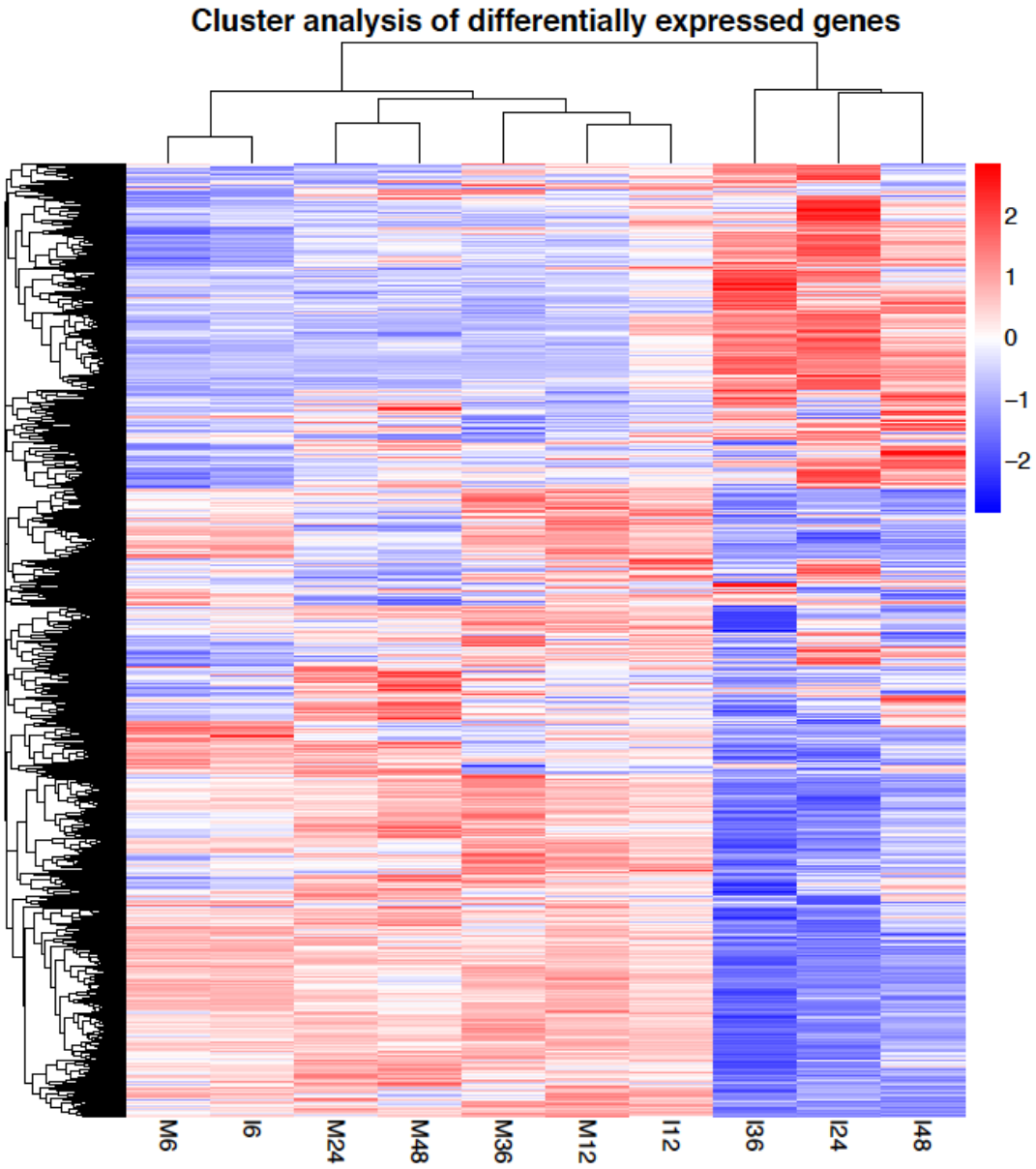

Figure 4.1. Cluster analysis of differentially expressed genes between *Phytophthora cinnamomi* inoculated and mock *Nicotiana benthamiana* leaves at 6, 12, 24, 36, and 48 h post inoculation (hpi). I6, I12, I24, I36, and I48 indicate *P. cinnamomi* inoculated leaves, and M6, M12, M24, M36, and M48 indicate mock leaves.

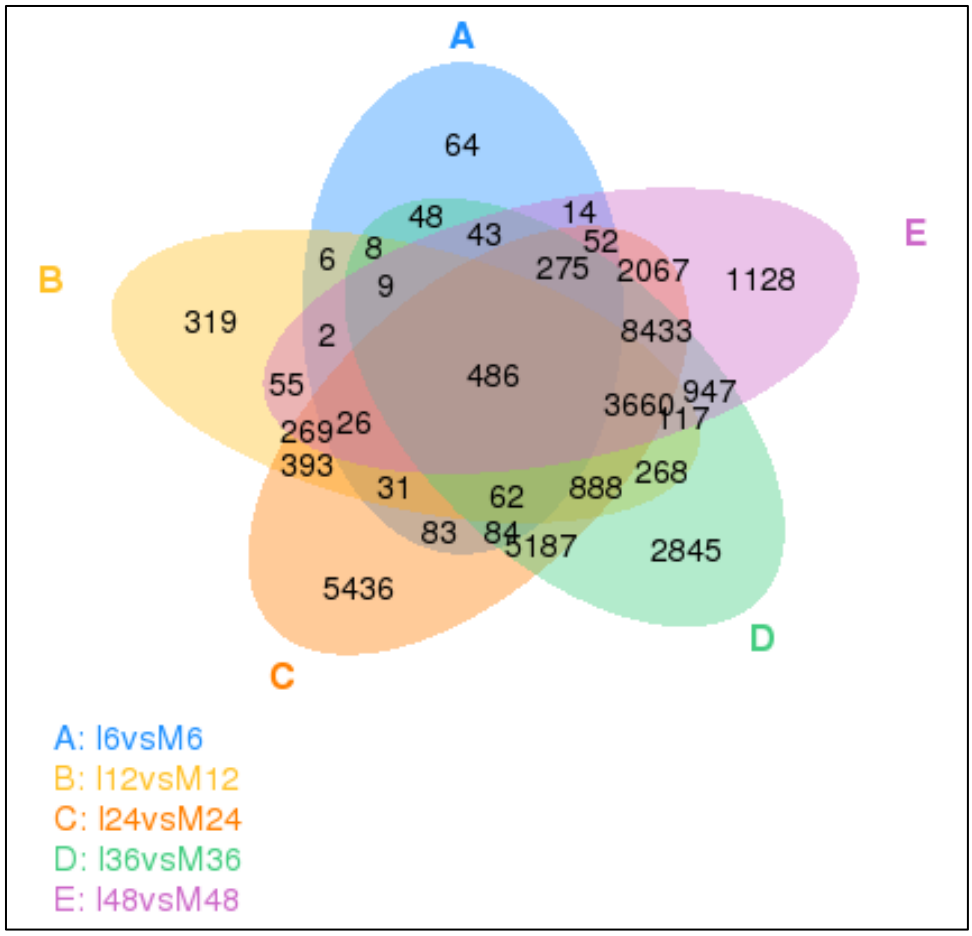

Figure 4.2. Venn diagram presenting the number of differentially expressed genes between *Phytophthora cinnamomi* inoculated and mock *Nicotiana benthamiana* leaves at each time point. **A,** I6 vs M6 h post inoculation (hpi), **B,** I12 vs M12 h post inoculation, **C,** I24 vs M24 h post inoculation, **D,** I36 vs M36 h post inoculation, and **E,** I48 vs M48 h post inoculation. 64 DEGs are uniquely expressed at 6 hpi, 319 at 12 hpi, 5436 at 24 hpi, 2845 at 36 hpi, and 1128 at 48 hpi. The sum of the number in the circle presents the total number of differentially expressed genes, and the overlap presents the differentially expressed genes in common.

Functional category			6hpi	12hpi	24hpi	36hpi	48hpi
Hormone signaling	SA	Phenylalanine ammonia-lyase (PAL)	0.5	2.8	3.5	4.7	5.8
		SA binding			-5	-10.5	
	JA	Allene oxide synthase			7	9	6
		JA-12-oxophytodienoate reductase			5.8	5.9	5
		Ketoacyl CoA thiolase			4.9	1.2	3.7
		F-box proteins	2.3		4.2	1.1	8.7
		Cytochrom P450 (CYP)	3	3.9	8.4	13.3	6.8
	ET	S-adenosylmethionine synthase			4	4.5	
		Ethylene proteinase inhibitor	-1.4				
Transcription factors	Ethylene response transcription factor		7.2	8	9.5	10	
	WRKY transcription factor 51 (WRKY51)		3.4	6.8	11.2	12.2	10.8
	Pathogenesis related gene transcription activator		4.4	6	8	13.3	
	Heat stress transcription factor					11.5	12
PR genes	PR1-Basic form of pathogenesis related protein 1			5.6	10	12	13
	PR4-Chitin binding				10.2	11.6	10.8
	PR5-Thaumatin like protein				6.1	6.8	5.6
	PR9-Peroxidase		1.5	10.6	12.2	13	6.5
Receptor like kinase (RLK)	LRR receptor-like kinase		2	5	9.1	7.1	7.9
	Cysteine rich receptor like protein kinase		3.8	11.5	12	8.6	7.5
Antifungal	Premnaspirodiene oxygenase (HPO)			12.3	19.3		19
	Aristolochene synthase (EAS)		4.3	3.4	17	12.7	11.2

Figure 4.3. Heat map of the gene Ontology-based functional categorization of differently expressed genes between *Phytophthora cinnamomi* inoculated and mock *Nicotiana benthamiana* leaves at 6, 12, 24, 36, and 48 h post inoculation (hpi) based on the log2 fold change values. Expression differences are shown in different colors. Red means down regulation and green means up regulation. Fold differences for WRKY transcription factors and cytochrome P450 for all time points were calculated by taking the mean fold-difference of all represented genes. Phenylalanine ammonia-lyase fold difference was calculated by taking the mean fold difference for 24, 36, and 48 hpi. For allene oxide synthase, S-adenosylmethionine synthase, and ethylene-responsive transcription factor the fold differences were calculated by taking the mean fold difference at 24, 36, and 24 hpi, respectively. A complete list of representative DEG's can be found in the Supplemental Tables 4.1, 4.2, and 4.3.

KEGG pathway category			6hpi	12hpi	24hpi	36hpi	48hpi
Up-regulated	Metabolism and energy	Carbon metabolism	0	102	0	0	0
		Endocytosis	0	70	0	140	0
		Glycolysis / Gluconeogenesis	0	51	0	0	99
		Citrate cycle (TCA cycle)	0	40	0	0	0
		Glutathione metabolism	11	0	0	0	0
		Oxidative phosphorylation	0	0	0	0	132
	Biosynthesis	Biosynthesis of secondary metabolites	72	308	0	0	0
		Phenylpropanoid biosynthesis	26	70	0	0	0
		Terpenoid backbone biosynthesis	11	114	0	0	0
		Flavonoid biosynthesis	10	25	0	0	0
		Stilbenoid, diarylheptanoid, and gingerol biosynthesis	9	23	0	0	0
		Sesquiterpenoid and triterpenoid biosynthesis	9	12	0	0	0
		Biosynthesis of amino acids	0	88	0	0	0
	Plant-pathogen interactions	Plant-pathogen interaction	21	111	0	160	142
		Phagosome	0	0	0	88	0
	Transcription, translation, and replication	Protein processing in endoplasmic reticulum	20	0	0	0	189
		Ribosome	0	0	0	0	321
Down-regulated	Metabolism and energy	Photosynthesis	13	0	0	79	0
		Photosynthesis-antenna proteins	8	0	0	0	0
		Ether lipid metabolism	7	0	0	0	0
		Linoleic acid metabolism	5	0	0	0	0
		Carbon metabolism	0	0	0	167	127
		Carbon fixation in photosynthetic organisms	0	0	79	79	65
		Glyoxylate and dicarboxylate metabolism	0	0	67	61	55
		Metabolic pathways	0	0	0	0	770
		Glycine, serine, and threonine metabolism	0	0	0	0	45
		Porphyrin and chlorophyll metabolism	0	0	0	0	37
		Circadian rhythm - plant	0	0	0	0	31
	Biosynthesis	Carotenoid biosynthesis	0	0	0	0	31
		Valine, leucine, and isoleucine biosynthesis	0	0	0	0	21
	Plant-pathogen	Plant hormone signal transduction	0	57	0	0	139
	Transcription, translation, and replication	Ribosome	0	72	0	0	0
		Spliceosome	0	29	0	131	0

Figure 4.4. Heat map of major up- and down-regulated genes between *Phytophthora cinnamomi* inoculated and mock *Nicotiana benthamiana* leaves at five time points categorized by Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway. Expression differences are shown in different colors. Red means down regulation and green means up regulation.

Mahomed and Van den Berg, 2011; Avocado	Up-regulated
Thaumatin	✓
PR10-pseml	
Cytochrom P450-like TBP	✓
Metallothionein-like protein	✓
Profilin-like gene	
MLO transmembrane encoding gene	✓
Universal stress protein	✓
Reeksting et al., 2014; Avocado	**Up-regulated**
WRKY transcription factor (WRKY)	✓
Phenylalanine ammonia-lyase (PAL)	✓
B-glucanase	
Allene oxide synthase	✓
Allene oxide cyclase	✓
Oxophytodienoate reductase	✓
3-ketoacyl CoA thiolase	✓
F-box proteins	✓
S-adenosylmethionine synthase	✓
Isoflavone reductase	✓
Glutathione s-transferase	✓
Cinnamyl alcohol dehydrogenase	✓
Cinnamoyl-CoA reductase	✓
Cysteine synthase	✓
Quinone reductase	
NPR1	✓
Santos et al., 2017; Chestnut	**Up-regulated**
Leucine-Rich Repeat Receptor-Like Kinase (LRR-RLK)	✓
C2CD	
WRKY31	✓
ABR1	✓
Myb4	✓
RNF5	✓
PE-2	
Ginkbilobin2 (GNK2)-like	
Meyer et al., 2016; Eucalyptus	**Up-regulated**
WRKY75	✓
PR3	
NRT2.5	✓
MLO	✓
LRR-RLK7	✓
PR1-Basic form of pathogenesis related protein 1	✓
PR5-Thaumatin like protein	✓
GNK2-like	

Figure 4.5. Published defense genes up-regulated in response to *Phytophthora cinnamomi* infection on the roots of avocado, chestnut, and *Eucalyptus*. Genes with a check were similarly up-regulated in the *Nicotiana benthamiana* detached leaf pathosystem. Literature reviewed are: Mahomed and Van den Berg 2011 and Reeksting et al. 2014 for avocado, Santo et al. 2017 for chesnut, and Meyer et al. 2016 for *Eucalyptus*.

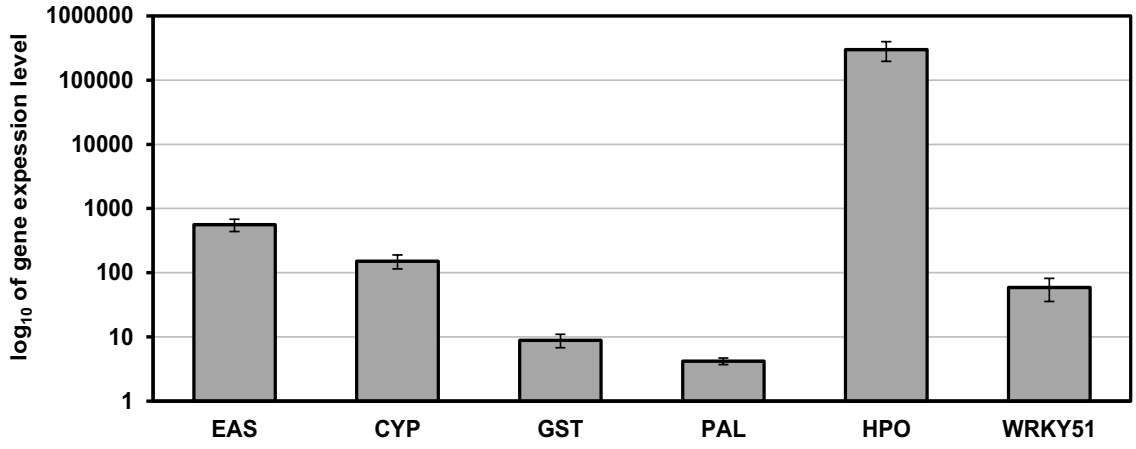

Figure 4.6. Gene expression patterns of six plant defense gene on *Phytophthora cinnamomi*-inoculated and mock-inoculated *Nicotiana benthamiana* leaves using the quantitative PCR (qPCR) technique. The six target genes are aristolochene synthase (EAS), cytochrome P450 (CYP), glutathione S-transferase (GST), phenylalanine ammonia-lyase (PAL), another cytochrome P450, premnaspirodiene oxygenase (HPO), and WRKY transcription factor 51 (WRKY51). Gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ (Ct = cycle threshold) comparative method. The error bars represent the standard error of three replicates.

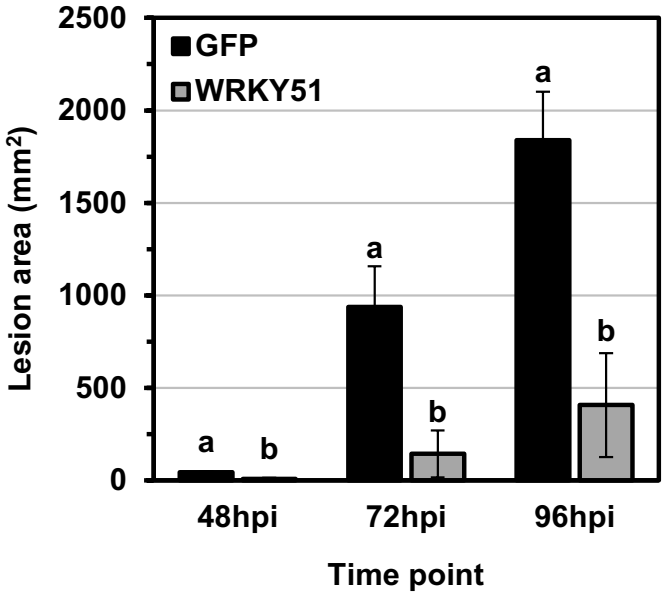

Figure 4.7. Lesion area on *Nicotiana benthamiana* overexpressing WRKY51 transcription factor (WRKY51) and green fluorescent protein (GFP) at 48, 72, and 96 h post inoculation (hpi) with *Phytophthora cinnamomi*. The error bars represent the standard error of three replicates. Bars followed by the same letter do not differ significantly according to Tukey's Honest Significant Difference test at $P = 0.05$.

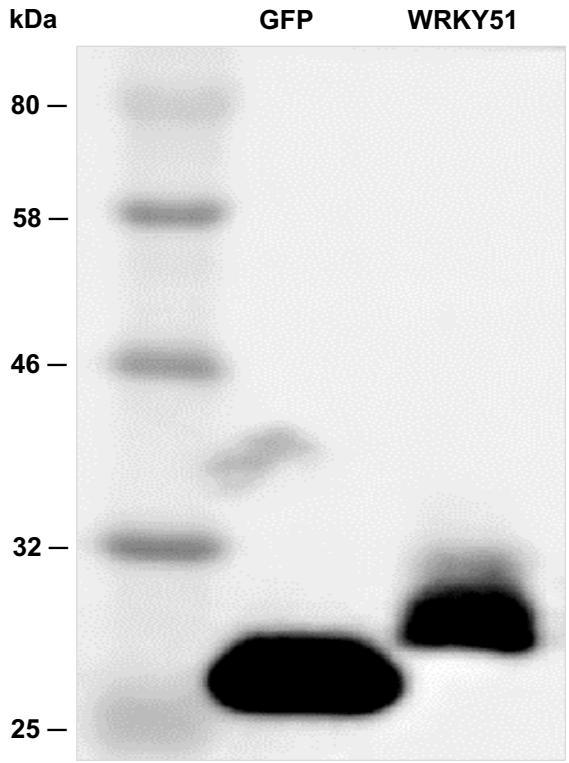

Figure 4.8. Western blot confirming the expression of the transiently overexpressed WRKY51 transcription factor (WRKY51) and green fluorescent protein (GFP) proteins in *Nicotiana benthamiana.* The WRKY51 was at the expected molecular weight of 28.0 kilodaltons (kDa). Green Fluorescent Protein (GFP) was confirmed at the expected molecular weight of 27.0 kDa.

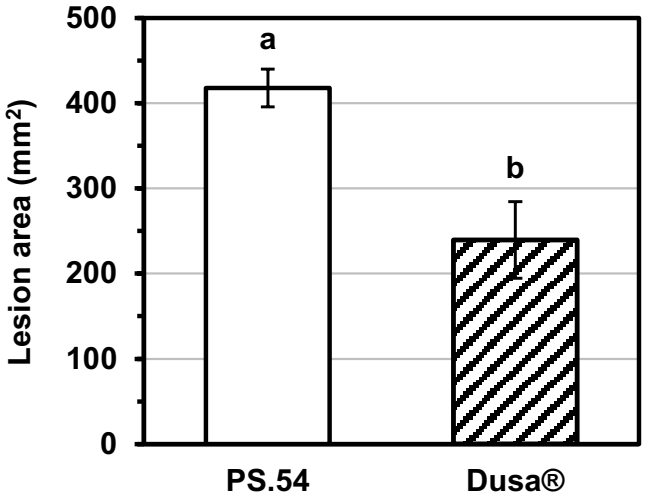

Figure 4.9. Lesion area on avocado leaves of PS.54 (susceptible) and Dusa® (moderate resistant) rootstocks 24 h post inoculation (hpi) with *Phytophthora cinnamomi* using a detached leaf assay. The error bars represent the standard error of five replicates. Bars followed by the same letter do not differ significantly according to Tukey's Honest Significant Difference test at $P = 0.05$.

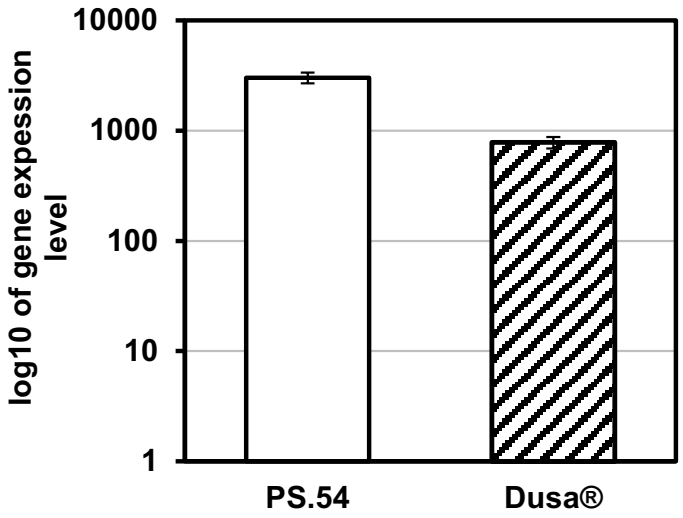

Figure 4.10. Gene expression of cytochrome P450 gene in avocado susceptible (PS.54) and moderate resistant (Dusa®) rootstocks at 24 h post inoculation (hpi) with *Phytophthora cinnamomi*. Gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ (Ct = cycle threshold). The error bars represent the standard error of three replicates.

Supplementary Table 4.1. The major up-regulated genes related to WRKY transcription factors between *Phytophthora cinnamomi* inoculated and mock *Nicotiana benthamiana* leaves at 6, 12, 24, 36, and 48 h post inoculation (hpi)

Time point	Gene accession number[x]	Annotation[y]	Log_2 fold difference
6 hpi	Niben101Scf01942g04001.1	WRKY51	Inf[z]
	Niben101Scf04011g05006.1	WRKY42	3.37
12 hpi	Niben101Ctg14444g00003.1	WRKY45	Inf
	Niben101Scf01942g04001.1	WRKY51	Inf
	Niben101Scf04944g05002.1	WRKY40	Inf
	Niben101Scf05057g04016.1	WRKY51	12.18
	Niben101Scf02188g00002.1	WRKY75	6.66
	Niben101Scf00530g06014.1	WRKY30	Inf
	Niben101Scf02511g04008.1	WRKY41	Inf
	Niben101Scf03850g00006.1	WRKY53	Inf
	Niben101Scf08526g02006.1	WRKY51	Inf
	Niben101Scf09644g00009.1	WRKY61	Inf
	Niben101Scf24496g01001.1	WRKY72	Inf
	Niben101Scf18774g02001.1	WRKY75	5.8
	Niben101Scf08321g01005.1	WRKY41	5.69
	Niben101Scf03348g00010.1	WRKY70	5.31
	Niben101Scf10306g00003.1	WRKY50	5.08
24 hpi	Niben101Ctg14444g00003.1	WRKY45	Inf
	Niben101Scf01942g04001.1	WRKY51	Inf
	Niben101Scf08526g02006.1	WRKY51	Inf
	Niben101Scf05057g04016.1	WRKY51	13.47
	Niben101Scf04944g05002.1	WRKY40	12.16
	Niben101Scf24496g01001.1	WRKY72	11.12
	Niben101Scf02188g00002.1	WRKY75	10.55
	Niben101Scf02511g04008.1	WRKY41	10.51
	Niben101Scf05166g02006.1	WRKY72	9.47
36 hpi	Niben101Scf05057g04016.1	WRKY51	12.71
	Niben101Scf24496g01001.1	WRKY72	12.41
	Niben101Scf01942g04001.1	WRKY51	12.25
	Niben101Ctg14444g00003.1	WRKY45	11.22
48 hpi	Niben101Scf05057g04016.1	WRKY51	10.77
	Niben101Scf01942g04001.1	WRKY51	Inf

[x] *Nicotiana benthamiana* gene ID in the reference genome (https://solgenomics.net/organism/Nicotiana_benthamiana/genome) (Bombarely et al. 2012).

[y] Annotations obtained by Blasting in Swiss-Prot Protein database (https://www.ebi.ac.uk/uniprot).

[z] Inf indicates read count is 0 in mock sample.

Supplementary Table 4.2. The major up-regulated genes related to cytochrome P450 between *Phytophthora cinnamomi* inoculated and mock *Nicotiana benthamiana* leaves at 6, 12, 24, 36, and 48 h post inoculation (hpi)

Time point	Gene accession number[x]	Annotation[y]	Log_2 fold difference
6 hpi	Niben101Scf00360 g00008.1	SOLCH Cytochrome P450 71D7 OS=*Solanum chacoense*	4.88
	Niben101Scf00158 g04018.1	ARATH Cytochrome P450 84A1 OS=*Arabidopsis thaliana*	4.57
	Niben101Scf00077 g03005.1	ARATH Cytochrome P450 98A3 OS=*Arabidopsis thaliana*	3.50
	Niben101Scf08273 g02004.1	ARATH Cytochrome P450 84A1 OS=*Arabidopsis thaliana*	6.06
	Niben101Scf02232 g00001.1	ARATH Cytochrome P450 710A1 OS=*Arabidopsis thaliana*	1.13
	Niben101Scf01037 g10003.1	ARATH Cytochrome b561 and DOMON domain-containing protein At5g47530	2.68
	Niben101Scf03170 g04002.1	SOYBN Cytochrome P450 98A2 OS=Glycine max	2.43
	Niben101Scf03262 g01002.1	SOLME Cytochrome P450 76A1 (Fragment) OS=*Solanum melongena*	2.13
	Niben101Scf03265 g00012.1	TOBAC Cytochrome b5 OS=*Nicotiana tabacum*	0.67
	Niben101Scf10556 g00025.1	SOYBN Cytochrome P450 98A2	1.01
12 hpi	Niben101Ctg11882 g00001.1	SOYBN Cytochrome P450 82A4	Inf[z]
	Niben101Scf00222 g01002.1	ARATH Cytochrome P450 82C4	7.86
	Niben101Scf09025 g00004.1	ARATH Cytochrome P450 94B3	Inf
	Niben101Scf08273 g02004.1	ARATH Cytochrome P450 84A1	6.67
	Niben101Scf00360 g00008.1	SOLCH Cytochrome P450 71D7	6.59
	Niben101Scf04011 g04001.1	PANGI Cytochrome P450 CYP736A12	6.06
	Niben101Scf00158 g04018.1	ARATH Cytochrome P450 84A1	5.02

	Niben101Scf00212g03032.1	ARATH Cytochrome P450 81D11	4.94
	Niben101Scf01037g10003.1	ARATH Cytochrome b561 and DOMON domain-containing protein At5g47530	4.76
	Niben101Scf01826g00001.1	ARATH Cytochrome P450 94B3	4.63
	Niben101Scf00077g03005.1	ARATH Cytochrome P450 98A3	4.42
	Niben101Scf03170g04002.1	SOYBN Cytochrome P450 98A2	4.40
	Niben101Scf07695g01020.1	PANGI Cytochrome P450 CYP72A219	4.39
	Niben101Scf03262g01002.1	SOLME Cytochrome P450 76A1 (Fragment)	4.02
	Niben101Scf06436g00006.1	SOYBN Cytochrome P450 78A3	3.15
	Niben101Scf00699g00006.1	PANGI Cytochrome P450 CYP72A219	3.15
	Niben101Scf01018g08001.1	SOYBN Cytochrome P450 98A2	3.14
	Niben101Scf14022g00011.1	ARATH Cytochrome P450 72A14	3.03
	Niben101Scf22099g00006.1	ARATH Cytochrome b561 and DOMON domain-containing protein At5g47530	2.89
	Niben101Scf05152g01001.1	ARATH Cytochrome b561 and DOMON domain-containing protein At3g25290	2.34
	Niben101Scf01943g08004.1	ARATH Cytochrome b561 and DOMON domain-containing protein At5g47530	1.96
	Niben101Scf10556g00025.1	SOYBN Cytochrome P450 98A2 OS=Glycine max	1.93
	Niben101Scf02232g00001.1	ARATH Cytochrome P450 710A1	1.89
	Niben101Scf10560g00010.1	SOYBN Cytochrome P450 98A2 OS=Glycine max	1.69
	Niben101Scf04930g00006.1	VICSA Cytochrome P450 94A1	1.50
24 hpi	Niben101Scf00109g09004.1	ARATH Cytochrome P450 94B3	Inf
	Niben101Scf01065g08001.1	PANGI Cytochrome P450 CYP736A12	Inf

	Niben101Scf01826g00006.1	ARATH Cytochrome P450 94B3	Inf
	Niben101Scf03674g01015.1	ARATH Cytochrome P450 94C1	Inf
	Niben101Scf04945g03014.1	PANGI Cytochrome P450 CYP72A219	Inf
	Niben101Scf09025g00001.1	ARATH Cytochrome P450 94B3	Inf
	Niben101Ctg11882g00001.1	SOYBN Cytochrome P450 82A4	14.50
	Niben101Scf00360g00008.1	SOLCH Cytochrome P450 71D7	12.36
	Niben101Scf00222g01002.1	ARATH Cytochrome P450 82C4 OS=*Arabidopsis thaliana*	11.46
	Niben101Scf01826g00001.1	ARATH Cytochrome P450 94B3	10.58
	Niben101Scf00158g04018.1	ARATH Cytochrome P450 84A1	10.25
	Niben101Scf00077g03005.1	ARATH Cytochrome P450 98A3	9.93
	Niben101Scf03170g04002.1	SOYBN Cytochrome P450 98A2	8.74
	Niben101Scf02232g00001.1	ARATH Cytochrome P450 710A1	7.62
	Niben101Scf03262g01002.1	SOLME Cytochrome P450 76A1	6.84
	Niben101Scf01037g10003.1	ARATH Cytochrome b561 and DOMON domain-containing protein At5g47530	6.63
	Niben101Scf00699g00006.1	PANGI Cytochrome P450 CYP72A219	6.56
	Niben101Scf14022g00011.1	ARATH Cytochrome P450 72A14	5.21
36 hpi	Niben101Scf00222g01002.1	ARATH Cytochrome P450 82C4	13.32
	Niben101Ctg11882g00001.1	SOYBN Cytochrome P450 82A4	13.28
	Niben101Scf01826g00001.1	ARATH Cytochrome P450 94B3	Inf
	Niben101Scf03674g01015.1	ARATH Cytochrome P450 94C1	Inf
48 hpi	Niben101Scf00360g00008.1	SOLCH Cytochrome P450 71D7	10.99

Niben101Ctg11882 g00001.1	SOYBN Cytochrome P450 82A4	10.21
Niben101Scf08535 g03001.1	ARATH Cytochrome P450 72A14	6.30
Niben101Scf01269 g02003.1	PICSI Cytochrome P450 716B1	Inf
Niben101Scf01826 g00001.1	ARATH Cytochrome P450 94B3	Inf
Niben101Scf03674 g01015.1	ARATH Cytochrome P450 94C1	Inf
Niben101Scf00222 g01002.1	ARATH Cytochrome P450 82C4	9.88
Niben101Scf00077 g03005.1	ARATH Cytochrome P450 98A3	9.12
Niben101Scf01065 g08001.1	PANGI Cytochrome P450 CYP736A12	8.74
Niben101Scf03170 g04002.1	SOYBN Cytochrome P450 98A2	8.02
Niben101Scf03262 g01002.1	SOLME Cytochrome P450 76A1 (Fragment)	7.07
Niben101Scf02232 g00001.1	ARATH Cytochrome P450 710A1	5.16
Niben101Scf00699 g00006.1	PANGI Cytochrome P450 CYP72A219	4.86
Niben101Scf04253 g02004.1	ARATH Cytochrome P450 710A1	4.75
Niben101Scf22099 g00006.1	ARATH Cytochrome b561 and DOMON domain-containing protein At5g47530	4.57
Niben101Scf01018 g08001.1	SOYBN Cytochrome P450 98A2	4.36
Niben101Scf07695 g01020.1	PANGI Cytochrome P450 CYP72A219	4.17
Niben101Scf10560 g00010.1	SOYBN Cytochrome P450 98A2	3.93

[x] *Nicotiana benthamiana* gene ID in the reference genome (https://solgenomics.net/organism/Nicotiana_benthamiana/genome) (Bombarely et al. 2012).

[y] Annotations obtained by Blasting in Swiss-Prot Protein database (https://www.ebi.ac.uk/uniprot).

[z] Inf indicates read count is 0 in mock sample.

Supplementary Table 4.3. The up-regulated genes other than those related to WRKY transcription factors and cytochrome P450 between *Phytophthora cinnamomi* inoculated and mock *Nicotiana benthamiana* leaves at 24, 36, or 48 h post inoculation (hpi)

Gene product	Time point	Gene accession number[x]	Annotation[y]	Log_2 fold difference
Phenylalanine ammonia-lyase	24 hpi	Niben101Scf12881g00009.1	sp|P45733|PAL3_TOBAC Phenylalanine ammonia-lyase	3.85
		Niben101Ctg06417g00002.1	sp|P45735|PALY_VITVI Phenylalanine ammonia-lyase (Fragment)	3.77
		Niben101Scf12881g00010.1	sp|P45733|PAL3_TOBAC Phenylalanine ammonia-lyase	3.43
		Niben101Scf03712g01008.1	sp|P25872|PAL1_TOBAC Phenylalanine ammonia-lyase	3.30
	36 hpi	Niben101Scf05488g00018.1	sp|P45735|PALY_VITVI Phenylalanine ammonia-lyase (Fragment)	Inf[z]
		Niben101Scf05617g00005.1	sp|P25872|PAL1_TOBAC Phenylalanine ammonia-lyase	5.29
		Niben101Scf04652g00007.1	sp|P25872|PAL1_TOBAC Phenylalanine ammonia-lyase	4.71
		Niben101Scf02432g00011.1	sp|P45733|PAL3_TOBAC Phenylalanine ammonia-lyase	4.26
	48 hpi	Niben101Scf05617g00005.1	sp|P25872|PAL1_TOBAC Phenylalanine ammonia-lyase	5.82
		Niben101Scf04652g00007.1	sp|P25872|PAL1_TOBAC Phenylalanine ammonia-lyase	5.82
Allene oxide synthase	24 hpi	Niben101Scf22689g00002.1	sp|P48417|CP74_LINUS Allene oxide synthase, chloroplastic OS=*Linum usitatissimum* GN=CYP74A PE=1 SV=1//2.07911e-76	8.69
		Niben101Scf10535g00001.1	sp|P48417|CP74_LINUS Allene oxide synthase, chloroplastic OS=*Linum usitatissimum* GN=CYP74A PE=1 SV=1//0	5.34
S-adenosylm-ethionine synthase	36 hpi	Niben101Scf02502g04001.1	sp|Q6SYB9|METK2_TOBAC S-adenosylmethionine synthase 2	5.11
		Niben101Scf11751g01007.1	sp|Q6SYB9|METK2_TOBAC S-adenosylmethionine synthase 2	3.96

Ethylene-responsive transcription factor	24 hpi	Niben101Scf12210g08011.1	sp|Q9FGF8|ABR1_ARATH Ethylene-responsive transcription factor ABR1	10.98
		Niben101Scf02242g03012.1	sp|Q9FGF8|ABR1_ARATH Ethylene-responsive transcription factor ABR1	7.90

[x] *Nicotiana benthamiana* gene ID in the reference genome (https://solgenomics.net/organism/Nicotiana_benthamiana/genome) (Bombarely et al. 2012).

[y] Annotations obtained by Blasting in Swiss-Prot Protein database (https://www.ebi.ac.uk/uniprot).

[z] Inf indicates read count is 0 in mock sample.

CHAPTER V. GENERAL CONCLUSIONS

Avocado production is expensive compared to many other crops in California. A shallow root system and their frequent placement on hillsides mean they need a lot of water to thrive. Ongoing drought conditions in California and sensitivity of avocado to saline conditions has forced some growers to abandon their avocado groves. These conditions combined with an aggressive pathogen population (e.g. *P. cinnamomi*) and limited treatment options necessitate an integrated disease management program, which is both economical and practical. Current treatment options for Phytophthora root rot (PRR) of avocado including: tolerant rootstocks, fungicide treatments (e. g. potassium phosphite and mefenoxam), and cultural practices such as mulching and careful watering, have become increasingly ineffectual in a time of drought and high disease pressure. Existing avocado rootstocks can be overcome by more virulent clonal pathogen populations. Also, the limited number of fungicides available results in a lack of rotation programs that increase the likelihood of fungicide resistance development, especially in areas where disease incidence is high and fungicide applications for avocado PRR are frequent.

In response to these challenges of avocado production in California, the specific research objectives addressed in the dissertation included:

i. Assessment of the phenotypic characteristics of several avocado *P. cinnamomi* isolates corresponding to two A2 clonal populations found in California, including *in vitro* mycelial growth rate, optimal growth temperature, sensitivities

to a few currently used and new Oomycota fungicides (e.g. mefenoxam and potassium phosphite, fluopicolide and oxathiapiprolin), and virulence to different avocado rootstocks.

ii. Develop and validate a detached leaf assay inoculation method using *Nicotianae benthamiana* to circumvent the difficulties associated with the avocado plant root inoculation method to assess the virulence of *P. cinnamomi* isolates.

iii. Establish baseline sensitivities of new Oomycota fungicides (e.g. ethaboxam, fluopicolide, mandipropamid, oxathiapiprolin) and *in vitro* sensitivities of mefenoxam and potassium phosphite to *P. cinnamomi* isolates representing the current pathogen population in California avocado growing areas.

iv. Evaluate the efficacy of the four new fungicides as compared to the two registered ones for the management of PRR of avocado seedlings and clonal rootstocks in the greenhouse.

v. Establish a model system on *N. benthamiana* to study plant defense gene expression in response to *P. cinnamomi* infection and validate differentially expressed defense genes using over-expression in the model system.

vi. Investigate the connections of the established *N. benthamiana-P. cinnamomi* model system and the avocado-*P. cinnamomi* pathosystem by comparing the behaviors of differentially expressed defense genes in the two systems.

This study provides new information on the phenotypic characteristics of *P. cinnamomi* populations in California avocado growing areas and management strategies

for PRR of avocado, as well as long term molecular tools to study host-*P. cinnamomi* interactions. Results and conclusions found through this project include:

i. *P. cinnamomi* isolates in two A2 clonal populations in California avocado growing regions, A2 clade I collected from 1989 to 2010 in northern regions and A2 clade II collected in 2009 and 2010 in southern regions, were characterized. The isolates from southern regions had a slower *in vitro* mycelial growth rate at 22°C, higher optimal growth temperature, were more virulent on both susceptible and tolerant avocado rootstocks, and were less sensitive to potassium phosphite compared to the isolates collected in northern avocado growing regions. The results suggest that a negative correlation might exist between the *in vitro* growth rates of some *P. cinnamomi* isolates with the level of virulence and sensitivity to potassium phosphite, which could potentially indicate a fitness cost for some traits over other acquired traits. This information will also useful for developing effective disease management strategies when dealing with these particular *P. cinnamomi* populations prevalent in some geographic locations.

ii. In the *N. benthamiana*-*P. cinnamomi* model system, the inoculum level of *P. cinnamomi* using the detached leave assays was evaluated using RT-qPCR. A positive correlation was found on the infection level between the *P. cinnamomi* inoculated *N. benthamiana* leaves and the pathogen infected avocado roots. The results suggested that *P. cinnamomi* inoculated detached leaf assays could be used as an alternative method to circumvent the difficulties of using avocado root inoculation methods to assess the virulence of large number of *P. cinnamomi*

isolates. The advantages of the detached leaf assay compared to the avocado root inoculations include: greater reproducibility, increased replication, more consistent delivery and localization of the inoculum in the leaves, uniform incubation conditions, more accurate quantification of pathogen, and a reduction of space required for inoculations.

iii. Baseline sensitivities of 71 isolates of *P. cinnamomi* representing the current pathogen population on avocado in California was established for four new Oomycota-targeting fungicides (e.g. ethaboxam, fluopicolide, mandipropamid, and oxathiapiprolin). The four new fungicides demonstrated high *in vitro* toxicity with relatively low mean EC_{50} values to the *P. cinnamomi* California population. The *in vitro* sensitivities for each of these compounds displayed a unimodal distribution and a narrow range of EC_{50} values for mycelial growth inhibition of 71 *P. cinnamomi* isolates. The narrow ranges in sensitivities among isolates with no distinct less-sensitive outliers in the distribution suggested a reduced potential for selection of resistance with the proper use of these fungicides. The baseline sensitivity ranges reported in this dissertation can be used as references in future monitoring for fungicide resistance in populations of the pathogen.

iv. The *in vitro* sensitivities of the 71 *P. cinnamomi* California isolates to mefenoxam and potassium phosphite were evaluated. The range of EC_{50} values for mefenoxam in this study displayed a unimodal distribution and a narrow range, suggesting the current usage pattern for this fungicide to control avocado PRR in California nurseries and orchards has not resulted in mefenoxam resistance in the

P. cinnamomi population. Alternately, a wide range of *in vitro* sensitivities to potassium phosphite with a bimodal distribution was detected for the 71 isolates, which separated the current pathogen population into two sensitivity groups indicating a shift in population sensitivity. Isolates with reduced sensitivity to potassium phosphite were identified. There was a significant difference in mean EC_{50} values between isolates from the southern and northern avocado growing regions. The higher value for isolates from southern California production areas may be due to higher field rates or more frequent applications of potassium phosphite to manage PRR in avocado orchards.

v. The four new fungicides were moderately to highly effective in reducing PRR and *P. cinnamomi* populations in the rhizosphere soil of the avocado seedlings and rootstocks in our greenhouse studies. Overall, oxathiapiprolin was the most effective among fungicides evaluated. These new fungicides were often significantly more effective than potassium phosphite or mefenoxam. Still, the efficacy of potassium phosphite was demonstrated with significant reductions in PRR on the seedlings and rootstocks. These results could explain why potassium phosphite is still effectively used in managing PRR in California since many growers cultivate avocado trees grafted on the tolerant Dusa® rootstock.

vi. RNAseq transcriptome analysis of infected *N. benthamiana* leaves at 6, 12, 24, 36, and 48 hours post-inoculation (hpi) was completed. Major biochemical pathways that were up-regulated included the biosynthesis of secondary metabolites and plant-pathogen interactions at 12 hpi. Major biochemical

pathways that were down-regulated in response to *P. cinnamomi* infection were metabolic pathways and carbon metabolism at 48 hpi. Differentially expressed defense genes were identified in hormone signaling, transcription factors, pathogenesis related, and resistance functional groups. Specifically, at 36 hpi, *cytochrome P450* (CYP), a jasmonic acid (JA) responsive gene, was up-regulated 13.3-fold and a salicylic acid (SA) binding gene was down-regulated 10.5-fold. *CYP* was also significantly up-regulated in infected avocado leaves, both Dusa® (tolerant) and PS.54 (susceptible), at 24 hpi.

vii. Transient over-expression of WRKY transcription factor 51 in detached *N. benthamiana* leaves successfully produced a resistance phenotype. At 96-hpi the mean lesion area of the WRKY51 inoculated leaves was 407 mm^2 compared to the GFP control leaves with a mean lesion area of 1838 mm^2. In most leaves' over-expression of this well described defense gene either completed inhibited lesion formation after inoculation with *P. cinnamomi* zoospores or delayed and decreased the size of the lesion. This supports the theory that RNAseq identified differentially expressed genes can influence the disease phenotype of this pathogen in functional assays.

The knowledge and tools developed through this research will have a significant impact on current and future management strategies for PRR on avocado. As a result of this work oxathiapiprolin will be available for the treatment of PRR on avocado in California in 2020. Avocado growing regions with a higher risk of resistance to current

treatments have also been identified increasing the urgency to use oxathiapiprolin in rotation with potassium phosphite in these areas. Finally, the *N. benthamiana* model system has increased our understanding of functionally important defense related genes differentially expressed in response to *P. cinnamomi* infection. In the future, RNAseq transcriptome analysis of inoculated *N. benthamiana* roots can identify DEG's that are universally expressed and DEG's that are uniquely expressed in the roots. Virus Induced Gene Silencing (VIGS) can be used to knock-down expression of identified defense genes in the roots of *N. benthamiana* for functional validation. As the annotated avocado genome is developed it will be possible to identify homologs in avocado to functionaly validated universally expressed defense genes in our model system. Functional assays developed for avocado will focus on these identified homologs.