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Etiology and Epidemiology of Wood Diseases in Nut Crops and the Impact of Deficit Irrigation on Tree Productivity

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Dedication

This dissertation is dedicated with love to my parents Alicia Luna and Adolfo Santiago who I will always be grateful for instilling in me good values to pursue a career in science and fortitude to confront life challenges.

ABSTRACT OF THE DISSERTATION

Etiology and Epidemiology of Wood Diseases in Nut Crops
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by

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Doctor of Philosophy, Graduate Program in Plant Biology
University of California, Riverside, June 2021
Dr. Philippe Rolshausen, Chairperson

In recent decades, the production of nuts has increased due to the higher consumption demand globally. Almonds and walnuts are one of the most profitable crops worldwide. The emergence of fungal diseases (*Botryosphaeria* and *Diaporthe*) coupled with extreme weather conditions increase the vulnerability to diseases outbreaks and affect tree longevity. Therefore, it is important to elucidate the etiology of diseases, develop disease management strategies and understand how water stress impact tree longevity in mature and young almond and walnut orchards.

The following studies presents i) the etiology of wood diseases in Chilean production; ii) the epidemiology of the causal agents of wood diseases in California almond and walnut orchards; and iii) the effect interaction between water stress and wood pathogen interaction on the physiology, morphology, and water potential on English walnut trees.

The findings in Chile reveal the presence of three Botryosphaeriaceae (*Diplodia mutila*, *Diplodia seriata* and *Neofusicoccum parvum*) and two Diaportheaceae species

(*Diaporthe australafricana* and *Diaporthe cynaroidis*) in walnut orchards between Valparaiso and Maule region with *Neof. parvum* as the most virulent pathogen.

Our second study consisted of comparative profiling of fungal canker pathogens from spore traps and wood samples in almond and walnut orchards in California. Our findings show five genera in the Botryosphaeriaceae family, one genus of the family Diaportheaceae in both almond and walnut orchards along with the insect-vectored *Ceratocystis* as the major pathogen from almond trees. The data also shows that orchard age, wet events, and precipitation highly influenced disease severity.

Our last experiment consisted of determining the effects of water stress and wood pathogens on walnut tree physiology. Our results reveal that water deficit treatments severely affect physiological and morphological traits. Furthermore, as water stress progressed, mid-day water potential increased thus becoming a factor to infection by *Botryosphaeria* spp. As a consequence of this event, whole tree canopy photosynthesis was greatly reduced.

In summary, our studies show the importance of adopting integrated disease management strategies to minimize the level of disease incidence and incorporate the optimum water management to reduce infection of fungal trunk pathogens.

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General Introduction

Almond (*Prunus dulcis*) and walnut (*Juglans regia*) are commercially cultivated crops with high economic value worldwide. In 2020, California almond production reached 3.0 billion pounds with an estimate value of over $6 million in value (USDA 2020). In 2019, it was reported that 37% of almond production was exported to the European Union (CDFA-2019-2020). The main variety produced in CA is Nonpareil (39%), followed by Monterey (17%) and Butte/Padre (14%) (Almond Board CA 2019). The production of almond takes place mainly in Kern, Fresno, Stanislaus, Merced, and Madera counties in CA which all together contribute to two thirds of California's almond production (CDFA 2020).

California is also an important producer of walnuts with the most important variety as Chandler (37%), followed by Hartley (17%) and Howard (9%). The production is mainly located in the San Joaquin Valley and Sacramento (CDFA 2019). In 2019, CA reached over 326,500 metric tons of walnuts with a value over 1 billion USD (CDFA-2019-2020). This ranks CA as the second most important producer of walnuts (27%) worldwide after China (46%) (FAO 2019).

In South America, Chile is the current leader of walnut production. The English walnut cultivation is concentrated 94% commercially between Atacama and BioBio-region (ODEPA, 2020). In this production area, the dominant varieties are Chandler (70%) and Serr (24%). On the other hand, in the 2019/2020 season, Chile produced 139,266 tons of walnuts of which exported 123,370 tons profiting 594 million USD (ChileNut 2020; ODEPA 2020). The nut industry in Chile is experiencing a rapid and intense growth in

terms of volumes and surface due to the adoption of mechanized harvest. However, the damages caused in walnut trees, and especially wounding, has provided increased incidence of fungal pathogens leading to a decrease in yield in producing walnut orchards.

Almond and walnut are usually infected by a range of canker disease pathogens, which often leads to serious yield losses (Michailides et al. 1991). The family Botryosphaeriaceae and Diaportheaceae are present in different geographical and climatic areas and are important groups that encompasses a range of fungi that are either pathogens, endophytes or saprobes predominantly in a wide variety of woody hosts (agricultural and non-cultivated plants). They affect vascular and cortical tissue (Michailides 1991; Michailides and Morgan 2004) and induced symptoms such as leaf blight, branch/shoot blight, leaf spot, dieback fruit-rot, root rot, and stem canker (Guarnaccia et al. 2018; Fang et al. 2020). Currently, there are over 193 species of *Botryosphaeria* which 17 genera have been recognized phylogenetically (Phillips et al. 2013) and about 150 *Diaporthe* species supported by molecular data (Gomes et al. 2013, Lombard et al. 2014).

Worldwide several species of *Botryosphaeria* (*Botryosphaeria dothidea*, *Diplodia mutila*, *Dip. seriata*, *Dothiorella iberica*, *Lasiodiplodia citricola*, *Neofusicoccum mediterraneum*, *Neof. nonquaesitum*, *Neof. parvum*, *Neof. vitifusiforme*, *Neoscytalidium dimidiatum*) have been reported in nut crops (Chen et al. 2014; Li et al. 2015; Sohrabi et al. 2020). Both California and Chile experience a Mediterranean climate suitable for walnut production but also vulnerable to drought events which could be a major predisposing factor to infection. Although similar pathogens have been reported in California and Chile

in woody crops, it is still unknown whether they are different strains with similar degree of virulence.

The family Diaportheaceae is also well-known worldwide as disease causing agents in many economically important crops including avocado (Guarnaccia et al. 2016), citrus (Guarnaccia and Crous, 2017) grapes (Van Niekerk et al. 2005; Lawrence et al. 2015) and walnuts (Chen et al. 2014) causing similar symptoms of *Botryosphaeria* (Lawrence et al. 2015). However, Diaportheaceae species in nut crops have been identified as less aggressive than Botryosphaeriaceae species (Chen et al. 2014). Several species of *Diaporthe* have been identified in walnut in CA (Chen et al. 2014), China (Fan et al. 2018), Spain (López-Moral et al. 2020), and recently in Chile (Jiménez-Luna et al. 2020).

Botryosphaeria and *Diaporthe* spp. enter wounds through mechanical means such as grafting, pruning, and by natural wounds caused from splitting of branches as a consequence of strong winds or frost damage (Hartill and Everett 2002). The buildup of inoculum during suitable environment can quickly grow and disseminate during rain or irrigation events. The initial process of penetration requires the dispersal and attachment of spores to the host. Once the spores attach to external surfaces of the host, they penetrate, remain dormant, germinate, and cause infection under favorable environmental conditions attacking mainly the vascular system (e.g., xylem and phloem) (Agustí-Brisach et al. 2019; Michailides and Hassey 2010; Pouzoulet et al., 2014).

The frequent events of drought and water scarcity become a limiting factor for perennial tree's growth, yield, and survival (Vahdati and Lotfi 2013). Studies in crops systems have documented how drought negatively impacts plant physiology (e.g., closure

of stomates to preserve water) (Knipfer et al. 2020; Sadat-Hosseini et al. 2019; Yordanov et al. 2000). Water stress has become an important limiting factor to crop productivity specifically to the English walnut (*Juglans regia*) since it is relative sensitive to water deficit (Vahdati and Lofti 2009). As walnut planted acreage continues expanding in California, the competition for water resources increases causing limited water supply. Therefore, there is the need to adopt deficit irrigation strategies on walnut production with the goal of sustaining and obtaining maximum nut yield while saving water specially during drought events. In almonds, it is established that resuming irrigation in the post-harvest is crucial since water stress during this stage will cause yield reduction the following season (Esparza et al. 2001). However, the application of deficit irrigation in walnut has not been as successful and effective as in other perennial crops such as almonds, pistachio, and wine grape due to the fact that water shortage in any development stage will affect productivity and nut quality (UC-ANR 2015). Applying water stress or causing leaf defoliation as a result of underirrigation could affect all phases of nut and tree development for the following seasons. The key point is to apply a beneficial water deficit irrigation that will reduce the interruption of transpiration and photosynthesis and the effect of nut and tree development (UC-ANR 2015).

In addition, the plant response to a combination of abiotic and biotic interaction at the whole plant and crop level is a complex integration of stress effects and responses over time. However, it is still unknown how walnut trees sustain production when exposed to a combination of stress; water limiting conditions and fungal attack. Nonetheless, it is important to provide insights of how plant's physiological performance of walnut trees is

impacted under different water regimes along with pathogen infection, and the future challenges that the current crop will face in extreme environmental conditions.

It is possible to minimize disease incidence and reduce nut yield loss by implementing an efficient integrated disease management with optimization of irrigation practices, use of resistant rootstocks and timely application of fungicides. However, to facilitate this approach it is required the knowledge of the biology of the pathogens, their geographic location, host specificity, aggressiveness in young vs. mature tissue and how water stress influences in tree and nut development. Therefore, the objectives of the following study were to: (i) identify and test the pathogenicity of *Botryosphaeria* and *Diaporthe* species from *Juglans regia* in Chile; (ii) assess and compare the diversity of fungal canker pathogens from spore traps and symptomatic wood samples within almond and walnut orchards in California, and (iii) evaluate the effects of water deficit and pathogen interaction on physiological and morphological traits, midday water potential and canopy carbon-water relations of young walnut trees.

References

Agustí-Brisach, C., Moral, J., Felts, D., Trapero, A., and Michailides, T. 2019. Interaction between *Diaporthe rhusicola* and *Neofusicoccum mediterraneum* causing branch dieback and fruit blight of English walnut in California, and effect of pruning wounds to the infection. *Plant Disease* 103:1196-1205

Almond Board of California annual report 2019. https://www.almonds.com/sites/default/files/2020-04/2019_Almanac.pdf

California Agricultural Exports 2019-2020. CDFA. Available at: https://www.cdfa.ca.gov/Statistics/PDFs/2020_Exports_Publication.pdf

California Almond Forecast. 2020. United States Department of Agriculture National Agriculture Statistics Service. https://www.nass.usda.gov/Statistics_by_State/California/Publications/Specialty_and_Other_Releases/Almond/Forecast/202005almpd.pdf

Chen, S., Morgan, D., Hasey, J., Anderson, K., Michailides, T. 2014. Phylogeny, Morphology, Distribution, and Pathogenicity of Botryosphaeriaceae and Diaportheaceae from English Walnut in California. *Plant Disease* 98: 636-652. 10.1094/PDIS-07-13-0706-RE.

ChileNut 2020. Asociación de Productores y Exportadores de Nueces en Chile. http://www.chilenut.cl

Esparza, G., DeJong, T. M., Weinbaum, S. A. 2001. Effects of irrigation deprivation during the harvest period on nonstructural carbohydrate and nitrogen contents of dormant, mature almond trees. *Tree physiology* 21(14): 1081–1086. https://doi.org/10.1093/treephys/21.14.1081

Fan, X., Yang, Q., Bezerra, J., Alvarez, L., Tian, C. 2018. *Diaporthe* from walnut tree (*Juglans regia*) in China, with insight of the *Diaporthe eres* complex. *Mycological Progress* 17. 10.1007/s11557-018-1395-4.

Fang, X., Qin, K., Li, J., Han, S., Zhu, T. 2020. Whole genome sequence of *Diaporthe capsici*, a new pathogen of walnut blight. *Genomics* 112. 10.1016/j.ygeno.2020.04.018.

Gomes, R., Glienke, C., Videira, S., Lombard, L., Groenewald, J. Z., Crous, P. W. 2013. *Diaporthe*: a genus of endophytic, saprobic and plant pathogenic fungi. *Persoonia* 31: 1–41. https://doi.org/10.3767/003158513X666844

Guarnaccia, V., Groenewald, J. Z., Woodhall, J., Armengol, J., Cinelli, T., Eichmeier, A., Ezra, D., Fontaine, F., Gramaje, D., Gutierrez-Aguirregabiria, A., Kaliterna, J., Kiss, L., Larignon, P., Luque, J., Mugnai, L., Naor, V., Raposo, R., Sándor, E., Váczy, K. Z., and Crous, P. W. 2018. *Diaporthe* diversity and pathogenicity revealed from a broad survey of grapevine diseases in Europe. *Persoonia* 40:135-153.

Guarnaccia, V. and Crous, P. 2017. Emerging citrus diseases in Europe caused by species of Diaporthe. *IMA Fungus* 8: 317–334. 10.5598/imafungus.2017.08.02.07.

Guarnaccia, V., Vitale, A., Cirvilleri, G., Aiello, D., Susca, A., Epifani, F., Perrone, G., Polizzi, G. 2016. Characterization and pathogenicity of fungal species associated with branch cankers and stem-end rot of avocado in Italy. *European Journal of Plant Pathology*. 146. 963-976. 10.1007/s10658-016-0973-z.

Hartill, W. and Everett, K. 2002. Inoculum sources and infection pathways of pathogens causing stem-end rots of 'Hass' avocado(Persea Americana). *New Zealand Journal of Crop and Horticultural Science* 30: 249-260. 10.1080/01140671.2002.9514221.

Jiménez Luna, I., Cadiz, F., Aravena, R., Larach, A., Besoain, X., Ezcurra, E., Rolshausen, P. E. 2020. First Report of *Diaporthe cynaroidis* and *D. australafricana* Associated with Walnut Branch Canker in Chile. *Plant Disease*. https://doi.org/10.1094/PDIS-01-20-0205-PDN

Knipfer, T., Reyes, C., Momayyezi, M., Brown, P. J, Kluepfel, D., & McElrone, A. J. (2020). A comparative study on physiological responses to drought in walnut genotypes (RX1, Vlach, VX211) commercially available as rootstocks. *Trees,* 34: 665-678. doi: 10.1007/s00468-019-01947-x

Lawrence, D., Travadon, R., Baumgartner, K. 2015. Diversity of Diaporthe species associated with wood cankers of fruit and nut crops in northern California. *Mycologia* 107: 926-940.

Li, G., Liu, F., Li, J., Liu, Q., Chen, S. 2015. Characterization of Botryosphaeria dothidea and *Lasiodiplodia pseudotheobromae* from English Walnut in China. *Journal of Phytopathology*. 10.1111/jph.12422.

Lombard, L., Van Leeuwen, GCM., Guarnaccia, V., Polizzi, G., Van Rijswick, P. C., Kaein, C. H. M., Rosendahl, J., Gabler, J., Crous, W. P. 2014. *Diaporthe* species associated with Vaccinium, with specific reference to Europe. *Phytopathologia Mediterranea* 53: 287–299.

López-Moral, A., Lovera, M., Raya, M., Cortés-Cosano, N., Arquero, O., Trapero, A., Agustí-Brisach, C. 2020. Etiology of Branch Dieback and Shoot Blight of English

Walnut Caused by Botryosphaeriaceae and *Diaporthe* Species in Southern Spain. *Plant disease* 104(2): 533–550. https://doi.org/10.1094/PDIS-03-19-0545-RE

Michailides, T. J. and Hasey, J. 2010. *Botryosphaeria* and *Phomopsis* cankers of walnuts in California. Walnut husk fly field meeting. University of California Cooperative Extension, Colusa, CA.

Michailides, T. J., Morgan, D. 2004. Panicle and Shoot Blight of Pistachio: A Major Threat to the California Pistachio Industry. APSnet Feature Articles. 10.1094/APSnetFeature-2004-0104.

Michailides, T. J. 1991. Pathogenicity, distribution, sources of Inoculum, and infection courts of *Botryosphaeria dothidea* on Pistachio. *Phytopathology* 81:566–573

ODEPA., 2020. Oficina de Estudios y Políticas Agrarias. Boletin de Nogal: producción, precios y comercio exterior. http://fedefruta.cl/odepa-repasa-las-cifras-anuales-de-2020-en-la-exportacion-de-fruta-fresca/

Phillips, A. J., Alves, A., Abdollahzadeh, J., Slippers, B., Wingfield, M. J., Groenewald, J. Z., & Crous, P. W. 2013. The Botryosphaeriaceae: genera and species known from culture. *Studies in mycology* 76(1): 51–167. https://doi.org/10.3114/sim0021

Pouzoulet, J., Pivovaroff, A. L., Santiago, L. S., Rolshausen, P. E. 2014. Can vessel dimension explain tolerance toward fungal vascular wilt diseases in woody plants? Lessons from Dutch elm disease and esca disease in grapevine. *Frontiers in plant science* 5: 253. https://doi.org/10.3389/fpls.2014.00253

Sadat-Hosseini, M., Vahdati, K., Boroomand, N., Arab, M., Asadi-Yekta, S. 2019. How walnut roots respond to drought stress: a morphological and anatomical study. 2068-7958.

Sohrabi, M., Mohammadi, H., León, M. et al. 2020. Fungal pathogens associated with branch and trunk cankers of nut crops in Iran. European Journal Plant Pathology 157: 327–351. https://doi.org/10.1007/s10658-020-01996-w

Vahdati, K., Lotfi, N. 2013. Abiotic stress tolerance in plants with emphasizing on drought and salinity stresses in walnut. pp.307-365. In: Abiotic Stress-Plant Responses and Applications in Agriculture. InTech.

Vahdati K, Lotfi N (2009) Abiotic stress tolerance in plants with emphasizing on drought and salinity stresses in walnut—chapter 10. In: Vahdati K (ed) Abiotic stress—plant responses and applications in agriculture, IntechOpen.

https://www.intechopen.com/books/abiotic-stress-plant-responses-and-applications-in-agriculture

van Niekerk, J., Groenewald, J. Z., Farr, D., Fourie, P., Halleen, F., Crous, P. 2005. Reassessment of Phomopsis species on grapevines. *Australasian Plant Pathology* 34: 27-39. 10.1071/AP04072.

Yordanov, I., Velikova, V., and Tsonev, T. 2000. Plant responses to drought, acclimation, and stress tolerance. *Photosynthetica* 38, 171–186. doi: 10.1023/A:1007201411474

Chapter I

Identity and Pathogenicity of Botryosphaeriaceae and Diaportheaceae Species from *Juglans regia* in Chile.

Abstract

English walnut (*Juglans regia*) has become one of the most important specialty crops in Chile and represents 11.5% of the total area of fruit trees, surpassed only by table grapes. As Chile expands walnut production rapidly, young tree plantations are at risk from the emergence of new fungal diseases affecting production. Fungi in the Botryosphaeriaceae and Diaportheaceae families have been recognized as main causes of walnut diseases globally with symptoms of dieback, canker, blight, and necrosis. The aims of this study were to survey young Chilean walnut orchards and profile the causal agents of wood diseases, measure their incidence and evaluate their aggressiveness. In winter 2017, thirteen young orchards were visited, and wood samples were collected from five orchards located in Valparaiso and Maule communes expressing dieback symptoms. Fungal isolates recovered were cultured, purified, characterized morphologically and representative isolates that were identified taxonomically with multi loci DNA sequencing (ITS, β-tubulin, and elongation factor). Phylogenetic analyses identified three *Botryosphaeria* species (*Neofusicoccum parvum*, *Diplodia mutila*, *Diplodia seriata*) and two *Diaporthe* species (*Diaporthe cynaroidis*, *Diaporthe australafricana*) and pathogenicity tests supported that *Neof. parvum* was the most virulent taxa. This investigation confirms the presence of pathogenic Botryosphaeriaceae and Diaportheaceae

in *J. regia* that should be considered a high risk for causing diseases in the young walnut industry in Chile.

Introduction

In the last decade, the production of the English walnut (*Juglans regia* L.) has increased rapidly, positioning China as the main producer worldwide (369,000 metric tons), followed by the USA (250,389 metric tons) (www.nutfruit.org). Chile has also become a major walnut producer, covering about 49,000 planted hectares, and attaining a production of 150,000 metric tons (Muñóz, 2017).

Botryosphaeriaceae has been reported as one of the major fungal groups adversely affecting walnut production globally, including California, USA (Chen et al. 2014), Spain (Moral et al. 2019b), China (Li et al. 2015) and Iran (Abdollahzadeh et al. 2013). Symptoms include dieback in buds, fruits and productive branches that subsequently lead to the death of the tree (Michailides et al. 2013). Several species pathogenic to walnut have been reported, including *Botryosphaeria dothidea*, *Bot. ribis*, *Botryodiplodia theobromae*, *Diplodia mutila*, *Dip. seriata*, *Dothiorella iberica*, *Dot. omnivora*, *Dot. sarmentorum*, *Lasiodiplodia citricola*, *Las. pseudotheobromae*, *Neofusicoccum mediterraneum*, *Neof. nonquaesitum*, *Neof. parvum*, *Neof. vitifusiforme*, *Neoscytalidium dimidiatum* (Chen et al. 2013a,b; Chen et al. 2014a,b; Chen et al. 2019; Eichmeier et al. 2020; Gusella et al. 2020; Haggag et al. 2007; Li et al. 2015; López-Moral et al. 2020; Rumbos 2007). *Las. citricola* and *Neof. parvum* (Chen el al. 2013a; Chen et al. 2014a,b) have been determined to be highly virulent to English walnut while *Dip. seriata* and *Dot. sarmentorum* are considered as the less aggressive (Chen et al. 2014a; López-Moral et al. 2020). Furthermore, *Neof.*

parvum has been reported as one of the most widely distributed taxa with over 90 hosts in more than 29 countries and 6 continents (Sakalidis et al. 2013; Gusella et al. 2020).

Diaportheaceae species were reported to be overall less aggressive to the English walnut than Botryosphaeriaceae species (Chen et al. 2014a; López-Moral et al. 2020). The symptoms caused by *Diaporthe* species include stem dieback and branch canker, shoot blight, leaf spots and fruit rot (Chen et al. 2014a; Li et al., 2019). *D. bicincta*, *D. eres*, *D. euonymi*, *D. juglandis*, *D. neotheicola*, *D. rhusicola*, *D. rostrata*, *D. rudis*, *Phomopsis albobestita*, *P. amygdali* and *P. arnoldiae* have all been reported in *Juglans* spp. across America, Europe, and Asia, (Uecker 1988; Anagnostakis 2007; Chen et al. 2014a; Gomes et al. 2013; Udayanga et al. 2014b, 2015; Fan et al. 2015a, 2018; López-Moral et al. 2020). *D. neotheicola* has been described as the most widespread pathogen in several hosts including walnut (Chen et al. 2014a; López-Moral et al. 2020).

In Chile, *Dip*. mutila, *D. australafricana* and *D. cynaroidis* were recently reported as the causal agent of canker and dieback in walnut orchards in Chile (Díaz et al. 2018; Jiménez Luna et al. 2020). Additionally, several studies have documented the presence of Botryosphaeriaceae and Diaportheaceae species on several other cultivated crops or native tree hosts including grapevine-*Vitis vinifera* (Valencia et al. 2015; Morales et al. 2012; Auger et al. 2004e), avocado-*Persea americana* (Valencia et al. 2019), highbush blueberry-*Vaccinium corymbosum* (Espinoza et al. 2009; Elfar et al. 2013), loquat-*Eriobotrya japonica*, kiwifruit-*Actinidia deliciosa* (Diaz et al. 2017; Palma et al. 2000), hazelnut-*Corylus avellana* L. (Guerrero et al. 2013), cranberry- *Vaccinium corymbosum* L.

(Guerrero et al. 1987), and native forest trees composed of *Araucaria araucana*, *Drimys winter*, and *Aristotelia chilensis* (Zapata et al. 2020).

A common avenue for both *Botryosphaeria* and *Diaporthe* species to infect trees is through spores airborne following rain that land on wounded tree surface caused by pruning, mechanical trunk shakers and wind injury (Moral et al. 2019a; Agustí-Brisach et al. 2019b; Moral et al. 2019b). Implementing cost-effective preventative practices that limit the incidence of these pathogens is key to long-term profitability of walnut orchards. The Institute of Agricultural Research INIA Chile has begun a research program with the ultimate goal of identifying fungal species involved in dieback and canker, and conduct fungicide tests to develop disease management strategies (Red Agricola, 2019). *Botryosphaeria* and *Diaporthe* species have been clearly established as a threat to walnut in several production areas globally and many species have already been found on alternate crops in Chile. The goal of this study is to establish the baseline of infection through an extensive survey in new walnut production areas in Chile and identify the taxonomic names of the species associated with walnut canker and dieback using phylogenetic analysis and confirm pathogenicity with standard bioassays.

Methods

Sampling locations and fungal collection.

In the winter 2017, thirteen walnut orchards ranging from five to fifteen years-old were surveyed in the central zone of Chile (two walnut orchards in commune V, four walnut orchards in commune VI, seven walnut orchards in commune VII). Only five walnut orchards (all cv. 'Chandler') showed symptoms of dieback: four orchards in commune VII and one orchard in commune V. Twenty-five symptomatic wood samples

were collected (five symptomatic trees × five samples/tree in each orchard). The fungal isolates obtained were from trees with necrotic stems and diseased shoots. All isolates displayed a dark to brown discoloration in the cortical and vascular tissue.

Symptomatic wood samples were disinfected with 1% sodium hypochlorite for 30 seconds and then rinsed in sterile water three times. Five wood chips (~3 mm^3) were placed in Petri dishes containing 2% PDA (Potato dextrose agar) medium supplemented with 0.2 g of tetracycline to suppress bacterial growth (Chen et al., 2014a; Morales et al., 2012). Pure fungal cultures were grown on 2% agar (Slippers et al. 2007) with the addition of sterile pine needles to stimulate sporulation and the formation of conidia so that cultures could be examined morphologically.

DNA extraction, PCR amplification and DNA sequencing.

Young mycelium covering an area of about 2 cm^2 from a pure culture was removed for DNA extraction. DNA was extracted using the Qiagen Blood and Tissue Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. PCR amplification was performed in 25 µL reaction volumes on a T100 thermal cycler (BioRad, Hercules, CA). Each reaction consisted of 17.4 µl sterile H_2O, 2.5 µL PCR buffer, 1 µl dNTPs (10 mM), 0.5 µL each primer (4 µM), 2 µL $MgCl_2$ (25 mM), and 0.1 µL *Taq* DNA polymerase (5 u/µL), with DNA added at 1-2 µL (10-20 ng DNA/µ1). The thermocycler setting consisted of initial denaturation at 94 °*C* for 2 min, then 35 cycles of the following three steps: 1 min at 94 °*C* for strand separation, 1 min at 58-65 ℃ for primer annealing and 1 min at 72 ℃ for amplification. The final extension step occurred for 3 min at 72 ℃. Amplicons were run on a 1% agarose gel using gel electrophoresis and stained with Gel Red dye (Biotium

Inc., Fremont, CA). The DNA regions amplified were the nuclear ribosomal internal transcribed spacer (ITS) region using the ITS1-ITS4 primer pair at 58 °*C* annealing temperature (White *et al*. 1990), the translation elongation factor 1-α gene (EF) using primers EF1-728F and EF1-986R at 58°*C* annealing temperature (Carbone et al. 1999), and the β-tubulin (TUB) gene using primers Bt2a and Bt2b at 65°*C* annealing temperature (Glass et al. 1995). Resulting bands were visualized under UV light using a Gel Doc Imager (Bio-Rad) and PCR products were purified using the QiaQuick PCR Purification Kit (Qiagen, Valencia, CA). Forward and reverse reads were generated by Sanger sequencing performed at the UCR Institute of Integrative Genome Biology sequencing core.

Phylogenetic analysis.

Forward and reverse reads of each DNA sample were edited and combined into a consensus sequence using Sequencher v. 5.0.1 (Gene Codes Corporation, Ann Arbor, MI). Sequences from each region were concatenated using Geneious v. 2020.1.1 (Biomatters Ltd., Auckland, New Zealand) and aligned manually. Sequence alignments and phylogenetic analyses were performed separately for Botryosphaeriaceae and Diaportheaceae owing to their taxonomic divergence. CBS type specimen and taxa identified from walnut and cultivated and wild plant hosts in Chile were used as reference sequences for phylogenetic reconstructions and all accession numbers are listed in Table 1. The nucleotide sequences of reference taxa were downloaded from the GenBank sequence database maintained by the National Center for Biotechnology Information (NCBI). The complete dataset of Botryosphaeriaceae consisted of 3 novel sequences and 28 reference sequences. The Diaportheaceae dataset consisted of 2 novel sequences and 51

reference sequences. Sequences were aligned using ClustalW implemented in MEGA 7 (Tamura et al. 2016) with manual adjustments. Phylogenetic trees for the Botryosphaeriaceae and Diaportheaceae were constructed using Maximum Likelihood with the optimal nucleotide substitution model determined by the Akaike Information Criterion (AIC; Akaike 1974). Nodal support consisted of nonparametric bootstrapping with 1,000 replicates. All positions containing gaps and missing data were eliminated. Separate phylogenetic analyses were performed for all fungal pathogens reported on alternate hosts (tree crops and wild hosts) in Chile for which DNA sequences were available on GenBank. These analyses were based on the ITS region alone.

In planta pathogenicity tests.

In winter 2019, a completely randomized design was set up to test the pathogenicity of the five fungal species isolated from walnut trees: *Neof. parvum*, *Dip. mutila*, *Dip. seriata*, *D. australafricana* and *D. cynaroidis*. The test was conducted at Pontificia Universidad Católica Valparaiso in Chile in an open field. The cultivar used for inoculation was 1.5-year-old 'Chandler' walnut tree grafted to a Vlach clonal rootstock. Pathogenicity tests were conducted on two sets of plants inoculated at different times for logistical reasons, with one set used for the three *Botryosphaeria* species and one set for the two *Diaporthe* species. A total of seventy plants were inoculated in total with ten trees for each treatment. Pathogenicity tests were repeated twice in 2017 and 2019. Inoculations were conducted by using 3 mm diameter mycelial PDA plugs from a 7-day pure culture. The stem wound was produced 30 cm above ground (half-way up the stem) and a 3 mm set was produced with a cork borer to insert an agar plug bearing mycelia. Negative controls

were inoculated with sterile 2% PDA plugs. After inoculation, the wounds were wrapped with parafilm. Data were recorded three months after inoculation by measuring canker length in the xylem. To complete Koch's postulate, each pathogen was re-isolated and cultured in 2% PDA medium. The presence of each pathogen was confirmed morphologically.

Statistical Analysis.

The data collected were analyzed using R. studio and depicted as box and whisker plots. The data were subjected to analysis of variance and the treatment means were compared using Tukey's least significant difference test at $P \leq 0.05$.

Results

Alignment of 31 DNA sequences from species in the Botryosphaeriaceae resulted in a dataset of 1299 nucleotide positions (557 positions in the ITS partition, 306 in the EF partition and 430 in the B-tubulin partition). These included 665 conserved sites (ITS = 439, EF = 116, B-tub = 110), 628 variable sites (ITS = 118, EF = 190, B-tub = 320) and 360 parsimony-informative sites (ITS = 104, EF = 170, B-tub = 86). The optimal model of nucleotide substitution inferred using the AIC was the Tamura-Nei model (Tamura and Nei 1993) with a discrete Gamma distribution (TN93 + G). The tree with the highest log likelihood (-6888.75) is shown in Figure 1.1. The phylogenetic analysis supported with strong bootstrap values the placement of our three Botryosphaeriaceae samples 301, 105 and 172 in the *Dip*. *mutila*, *Dip*. *seriata* and *Neof*. *parvum* clades respectively.

A separate phylogenetic analysis including ITS sequences of fungal samples previously identified from alternate hosts in Chile was also generated, because sequences

for all three loci were not always available in the NCBI database. Alignment of 53 DNA sequences of species in the Botryosphaeriaceae resulted in a dataset of 556 nucleotide positions. These included 412 conserved sites, 144 variable sites, 117 parsimony-informative sites and 27 singleton sites. The optimal model of nucleotide substitution inferred using the AIC was the Tamura-Nei model (Tamura and Nei 1993) with a discrete Gamma distribution (TN93 + G). The tree with the highest log likelihood (-2091.81) is shown in Figure S1.1. Results illustrate the diversity of pathogens in family Botryosphaeriaceae present in Chile and the range of crops affected by them. Sample 301 is one of four *Dip*. *mutila* isolates reported in Chile, the three others originating from apple, avocado and walnut. Sample 105 is one of four *Dip*. *seriata* isolates, the other three reported from apple, avocado, and grapevine. Sample 172 is one of three *Neof*. *parvum* isolates, the other two isolated from avocado and blueberry.

The alignment of 37 Diaportheaceae DNA sequences comprised 1764 nucleotide positions (580 ITS, 366 EF and 812 B-tub) of which 1035 were conserved (ITS = 451, EF = 105, B-tub = 479), 717 were variable (ITS = 129, EF = 259, B-tub = 329) and 617 were parsimony-informative (ITS = 94, EF = 239, B-tub= 284). The AIC-inferred optimal model of nucleotide substitution was a General Time Reversible model (Nei and Kumar 2000) with a discrete Gamma distribution (GTR + G). The tree with the highest log likelihood (-11260.77) is shown in Figure 1.2. Phylogenetic reconstruction placed our samples 302 and 102 in clades of *Diaporthe australafricana* and *D*. *cynaroidis*, respectively, albeit with moderate bootstrap support.

A separate phylogenetic analysis including ITS sequences of Diaporthaceae fungi previously identified from alternate hosts in Chile was also generated because the sequences for all three loci were not available in the NCBI database. It was based on an alignment of 53 Diaportheaceae DNA sequences comprising 570 nucleotide positions in the ITS region of which 387 were conserved, 183 were variable, 155 were parsimony-informative and 28 singleton sites. The AIC-inferred optimal model of nucleotide substitution was a General Time Reversible model (Nei and Kumar 2000) with a discrete Gamma distribution (GTR + G). The tree with the highest log likelihood (-3296.10) is shown in Figure S1.2. Our sample 102 is the only *D. cynaroidis* isolate reported in Chilean walnut at this time, and our sample 302 is one of four *D. australafricana* isolates, the other three originating from blueberry and kiwifruit.

The level of pathogen incidence in the five symptomatic walnut orchards sampled was measured as follows: *Neof.* parvum (100%), *Dip. mutila* (60%), *D. australafricana* (60%), *Dip. seriata* (40%) and *D. cynaroidis* (40%). *Neof. parvum* was the only fungal pathogen identified in commune V-Valparaiso. All five species were capable of causing wood lesions on inoculated walnut stems compared to the mock-inoculated control plants but with some significant differences in virulence (Figure 1.3) ($P<0.05$; One-way ANOVA followed by Tukey test for multiple comparison of means). *Neof. parvum* appeared to be the most aggressive species and caused significantly larger lesions than *Dip. mutila* ($P = 0.0045$). *Dip. seriata* was intermediate for wood lesion lengths but not significantly different from either *Neof. parvum* or *Dip. mutila* ($P = 0.0764$) (Figure 1.3A). In addition,

lesion length was not significantly different between *D. australafricana* and *D. cynaroidis* but significantly different from the mock-inoculated controls (Figure 1.3B).

Discussion

Our survey indicated that incidence of wood diseases in Chilean walnut orchards was still relatively low as only five of the thirteen orchards were symptomatic. This can be explained by the long incubation period required for wood pathogen to cause wood dieback (Duthie et al. 1991) coupled with the fact that walnut is a relatively young industry in Chile. The walnut industry started in Chile in the late 1970's with around 4,000 hectares which produced less than one metric ton per hectare. Over the years, Chile walnut production has grown to over 43,300 ha planted with a yield of 4.5MT/ha (INC 2021). Our study shows that several fungal species of *Botryosphaeria* and *Diaporthe* are already established in two important regions of walnut production in central Chile, suggesting that those may become increasingly problematic as orchards age and plantation size expand. Overall, this study confirms previous results from Díaz et al. (2018) and Jiménez Luna et al. (2020) that found *Dip. mutila*, *D. australafricana* and *D. cynaroidis* in walnut orchards and expand the host range of *Dip. seriata* and *Neof. parvum* to walnut in Chile.

The infection of young orchards with both *Botryosphaeria* and *Diaporthe* species could be attributed to different infection routes. One can propose that infections initially came from plant nursery materials as it has been reported in several crops (Chen et al. 2013b, Espinoza et al. 2009, Smit et al. 1996, Tennakoon et al. 2017, Whitelaw-Weckert et al. 2013) including walnut (Chen et al. 2013a). However, inoculum originated most likely from the alternate hosts grown at the proximity of walnut orchards that becomes

airborne following wet events (rain, sprinkler or furrow irrigation), which aids in the process of infection by dispersing spores, and infected exposed tree wounds caused by pruning or mechanical harvester (Luo et al. 2020, Michailides and Morgan 1993). In Chile all three *Botryosphaeria* species isolated from walnut have also been found to cause branch canker and dieback in avocado trees and walnut are often planted at the vicinity of avocado in the regions surveyed (Valencia et al. 2019). Moreover, those species were found on additional hosts including apple, blueberry and grapevine (Auger et al., 2004; Diaz et al. 2018; Espinoza et a., 2009; Morales et al. 2012), all of which have been grown locally and infected hosts can become source of inoculum for neighboring walnut orchards. Similarly, *D. australafricana* and *D. cynaroidis* have been identified in European hazelnut, blueberry and kiwifruit (Diaz et al., 2017; Elfar et al. 2013; Guerrero et al. 2013). Our phylogenetic analyses also indicated that several additional species of *Botryosphaeria* and *Diaporthe* known to be pathogenic to walnut such as *Las. theobromae, Dot iberica*, or *D. neotheicola* (Chen et al., 2014; Lopez-Moral et al., 2020; Sohrabi et al. 2020) have been reported on the same alternate hosts in Chile such as avocado (Valencia et al. 2019) and blueberry (Espinoza et al. 2008) and could potentially become a threat to local walnut production.

Our data supported that *Neof*. parvum, *Dip*. *Seriata, Dip*. *mutila*, *D*. *australafricana* and *D*. *cynaroidis* associated with walnut wood diseases were all pathogenic. This study is in line with previous studies indicating that *Neof*. *parvum* is one of the most aggressive wood pathogens to many crops in addition to English walnut (Chen et al. 2014a; Lopez-Moral., 2020), including almond (Inderbitzin et al., 2010; Holland et al. 2021), avocado (McDonald et al. 2011; 2009), citrus (Adesemoye et al. 2011) and grapevine (Úrbez-Torres

et al. 2009). The broad incidence and high virulence of *Neof. parvum* indicates that this fungus should be considered one of the main pathogens of walnut in Chile and could cause canker of trunk and limb eventually resulting in a decline of affected trees. *Dip. seriata* and *Dip. mutila* were weakly virulent with respect to wood lesions caused to walnut branches compared to *Neof. parvum* and those results are in accordance with other reports (Chen et al., 2014a; López-Moral et al., 2020). *D. australafricana* and *D. cynaroidis* were also in the same range of virulence as *Dip. seriata* and *Dip. mutila* and were comparable to previous published of mild aggressiveness of species in the *Diaporthe* group including *D. rhusicola* and *D. neotheicola* (Chen et al., 2014a; López-Moral et al., 2020). Genome sequence analyses for fungi causing wood diseases supported that *Diaporthe* species and *Diplodia* species lack the enzymatic capabilities to colonize woody tissues and break down cell wall lignin (Garcia et al. 2021; Morales-Cruz et al. 2015) and may be more responsible for shoot/fruit blights and twig dieback than capable of causing limb and trunk cankers as reported with *Neof. parvum*.

Protecting wounds with fungicide application is the best strategy to prevent fungal infections as demonstrated in other pathosystems (Diaz and Latorre, 2013; Rolshausen et al. 2020). Currently, in Chile the application of lime sulfur is used in walnut to control the development of *Botryosphaeria* sp. and *Diaporthe* sp. (SAG 2021). Integrated disease management remains one the main effective way to control fungi causing wood diseases. Pruning in dry weather, managing canopy size allowing ventilation and sunlight exposure, and maintaining low tree planting densities are recommended practices to minimize the risk and severity of infection (Moral et al., 2019a; Moral et al. 2019b). In addition, pruning

and removal of dead and infected tissues and avoiding excessive wetting of the trunk or canopy is strongly encouraged to limit the build-up spread of disease inoculum and extend crop longevity in productivity as shown in pistachio orchards and vineyards (Gispert et al., 2020; Michailides and Morgan 1993). To date there is no walnut cultivar known to be resistant to the causal agents of wood disease, although cv. 'Chandler' has been reported to be more tolerant to infection, followed by 'Tulare' and 'Vina' (Chen et al. 2014a). As Chile looks to expand its walnut production to meet global market demand, management of these diseases will be key to sustain longevity and productivity of orchards.

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References

Abdollahzadeh, J., Zare, R., Phillips, A. J. 2013. Phylogeny and taxonomy of *Botryosphaeria* and *Neofusicoccum* species in Iran, with description of *Botryosphaeria scharifii* sp. nov. *Mycologia* 105: 210–220.

Adesemoye, A.O., Eskalen, A. 2011. First report of *Spencermartinsia viticola*, *Neofusicoccum australe*, and *N. parvum* causing branch canker of citrus in California. *Plant Disease* 95:770.

Agustí-Brisach, C., Moral, J., Felts, D., Trapero, A., Michailides, T. J. 2019b. Interaction between *Diaporthe rhusicola* and *Neofusicoccum mediterraneum* causing branch dieback and fruit blight of English walnut in California, and effect of pruning wounds to the infection. *Plant Disease* 103:1196-1205

Anagnostakis, S.L. 2007. *Diaporthe eres* (*Phomopsis oblonga*) as a pathogen of butternut (*Juglans cinerea)* in Connecticut. *Plant Disease* 91:1198

Auger, J., M. Esterio, G. Ricke, and I. Pérez. 2004e. Black dead arm and basal canker of *Vitis vinifera* cv. Red Globe caused by *Botryosphaeria obtusa* in Chile. *Plant Disease* 88:1286.

Besoain, X., Fuentes, H. 1988. Cancrosis en níspero (*E. japonica*) causado *por Botryosphaeria dothidea* y su incidencia en la zona de Quillota. Simiente (Chile) 58(1-2):37.

Carbone, I., Kohn, M. L. 1999. A method for designing primer sets for speciation studies in filamentous ascomycetes. *Mycologia* 91(3):553-556.

Chen, S.F., Fichtner, E., Morgan, D. 2013a. First Report of *Lasiodiplodia citricola* and *Neoscytalidium dimidiatum* Causing Death of Graft Union of English Walnut in California. *Plant Disease* 97: 993-993. 10.1094/PDIS-10-12-1000-PDN.

Chen, S. F., Morgan, D., Beede, R. H., Michailides, T. J. 2013b. First report of *Lasiodiplodia theobromae* associated with stem canker of almond in California. *Plant Disease* 97:994.

Chen, S. F., Morgan, D. P., Hasey, J. K., Anderson, K., Michailides, T. J. 2014a. Phylogeny, morphology, distribution, and pathogenicity of Botryosphaeriaceae and Diaporthaceae from English walnut in California. *Plant Disease* 98:636-652.

Chen, S. F., Morgan, D. P., and Michailides, T. J. 2014b. Botryosphaeriaceae and Diaporthaceae associated with panicle and shoot blight of pistachio in California, USA. *Fungal Diversity* 67:157-179.

Chen, X., He, B., Li, Hao-Xi., Cernava, T., Sang, W., Yang, Mao-Fa., Yang, Wen-Jia. 2019. First Report of Black Rot on Walnut Fruits Caused by *Neofusicoccum parvum* in China. *Plant Disease* 103. 10.1094/PDIS-02-19-0387-PDN.

Díaz, G., Latorre, B., Ferrada, E., Gutierrez, M., Bravo, F., Lolas-Caneo, M. 2018. First Report of *Diplodia mutila* Causing Branch Dieback of English Walnut cv. Chandler in the Maule Region, Chile. *Plant Disease* 102. 10.1094/PDIS-11-17-1860-PDN.

Díaz, G., Mostert, L., Halleen, F., Lolas-Caneo, M., Gutierrez, M., Ferrada, E., Latorre, B. 2018. *Diplodia seriata* Associated with *Botryosphaeria* Canker and Dieback in Apple Trees in Chile. *Plant Disease* 103. 10.1094/PDIS-10-18-1785-PDN.

Díaz, G. A., Latorre, B. A., Lolas, M., Ferrada, E., Naranjo, P., Zoffoli, J. P. 2017. Identification and Characterization of *Diaporthe ambigua*, *D. australafricana*, *D. novem*, and *D. rudis* Causing a Postharvest Fruit Rot in Kiwifruit. *Plant disease* 101(8): 1402–1410. https://doi.org/10.1094/PDIS-10-16-1535-RE

Duthie, J. A., Munkvold, G. P., Marois, J. J. 1991. Relationship between age of vineyard and incidence of *Eutypa* dieback. *Phytopathology* 81: 1183.

Eichmeier, A., Pečenka, J., Špetík, M., Necas, T., Ondrášek, I., Armengol, J., Leon, M., Berlanas, C., Gramaje, D. 2020. Fungal Trunk Pathogens Associated with *Juglans regia* in the Czech Republic. *Plant Disease* 104: 761-771. 10.1094/PDIS-06-19-1308-RE.

Elfar, K., Torres, R., Díaz, G.A., Latorre, B.A. 2013. Characterization of *Diaporthe australafricana* and *Diaporthe* spp. Associated with Stem Canker of Blueberry in Chile. *Plant disease* 97(8): 1042–1050. https://doi.org/10.1094/PDIS-11-12-1030-RE

Eskalen, A., Faber, B., and Bianchi, M. 2013. Spore Trapping and Pathogenicity of Fungi in the Botryosphaeriaceae and Diaporthaceae Associated with Avocado Branch Canker in California. *Plant Disease* 97:329-332.

Espinoza, J., Briceño, E., Chavez, E., Úrbez-Torres, J. R., Latorre, B. 2009. *Neofusicoccum spp*. Associated with Stem Canker and Dieback of Blueberry in Chile. *Plant Disease* 93: 1187-1194. 10.1094/PDIS-93-11-1187.

Espinoza, J., Briceño, E., Latorre, B. 2008. Identification of species of *Botryosphaeria*, *Petalotiopsis* and *Phomopsis* in blueberry in Chile. APS Centennial Meeting.

Fan, X.L., Hyde, K.D., Udayanga, D., Wu, X.Y., Tian, C.M. 2015a. *Diaporthe rostrata*, a novel ascomycete from *Juglans mandshurica* associated with walnut dieback. *Mycological Progress* 14:1–8.

Fan, X., Yang, Q., Bezerra, J., Alvarez, L., Tian, C. 2018. *Diaporthe* from walnut tree (*Juglans regia*) in China, with insight of the *Diaporthe eres* complex. *Mycological Progress* 17. 10.1007/s11557-018-1395-4.

Garcia, J. F., Lawrence, D. P., Morales-Cruz, A., Travadon, R., Minio, A., Hernandez-Martinez, R., Rolshausen, P. E., Baumgartner, K. and Cantu, D. 2021. Phylogenomics of plant associated Botryosphaeriaceae species. *Frontiers in Plant Science* 18. DOI: 10.3389/fmicb.2021.652802.

Glass, N. L., Donaldson, G. C. 1995. Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. *Applied and environmental microbiology* 61(4): 1323–1330. https://doi.org/10.1128/AEM.61.4.1323-1330.1995

Gomes, R. R., Glienke, C., Videira, S. I., Lombard, L., Groenewald, J. Z., Crous, P. W. 2013. *Diaporthe*: a genus of endophytic, saprobic and plant pathogenic fungi. *Persoonia* 31: 1-41. https://doi.org/10.3767/003158513X666844

Guerrero, J., Godoy, I. 1987. Identificación y etiología de *Phomopsis vaccinii* Shear. Stevens y Bein en arándano (*Vaccinium corymbosum* L.). *Simiente* (Chile) 57(3):101.

Guerrero, J., Pérez, S. 2013. First Report of *Diaporthe australafricana*-Caused Stem Canker and Dieback in European Hazelnut (*Corylus avellana L.*) in Chile. *Plant disease* 97(12): 1657. https://doi.org/10.1094/PDIS-03-13-0286-PDN.

Gusella, G., Giambra, S., Conigliaro, G., Burruano, S., Polizzi, G. 2020. Botryosphaeriaceae species causing canker and dieback of English walnut (Juglans regia) in Italy. *Forest Pathology* 51. 10.1111/efp.12661.

Haggag, W. M., Abou Rayya, M., Kasim, N. E. 2007. First Report of a Canker Disease of Walnut Caused by *Botryodiplodia theobromae* in Egypt. *Plant disease* 91(2): 226. https://doi.org/10.1094/PDIS-91-2-0226B

Holland, L. A., Trouillas, F. P., Nouri, M. T., Lawrence, D. P., Crespo, M., Doll, D. A., Duncan, R. A., Holtz, B. A., Culumber, C. M., Yaghmour, M. A., Niederholzer, F. J. A., Lightle, D. M., Jarvis-Shean, K. S., Gordon, P. E., and Fichtner, E. J. 2020. Fungal pathogens associated with canker diseases of almond in California. *Plant Disease* 105(2): 346–360. https://doi.org/10.1094/PDIS-10-19-2128-RE

International Nut and Dried Fruit (INC). 2021. https://www.nutfruit.org/industry/publications/inc-magazine/articles/detail/industry-highlight-chilean-walnuts

Inderbitzin, P., Bostock, R. M., Trouillas, F. P., Michailides, T. J. 2010. A six-locus phylogeny reveals high species diversity in Botryosphaeriaceae from California almond. *Mycologia* 102:1350-1368.

Jiménez Luna, I., Cadiz, F., Aravena, R., Larach, A., Besoain, X., Ezcurra, E., Rolshausen, P. E. 2020. First Report of *Diaporthe cynaroidis* and *D. australafricana* Associated with Walnut Branch Canker in Chile. *Plant Disease*. https://doi.org/10.1094/PDIS-01-20-0205-PDN

Li, L., Mohd, M. H., Zakaria, L. 2019. Identification and pathogenicity of *Diaporthe* species associated with stem-end rot of mango (*Mangifera indica L.*). *European Journal of Plant Pathology*. 10.1007/s10658-019-01800-4.

Li, G., Liu, F., Li, J., Liu, Q., Chen, S. 2015. Characterization of *Botryosphaeria dothidea* and *Lasiodiplodia pseudotheobromae* from English Walnut in China. *Journal of Phytopathology*. 10.1111/jph.12422.

López-Moral, A., Lovera, M., Raya, M., Cortés-Cosano, N., Arquero, O., Trapero, A., Agustí-Brisach, C. 2020. Etiology of Branch Dieback and Shoot Blight of English Walnut Caused by Botryosphaeriaceae and *Diaporthe* Species in Southern Spain. *Plant disease* 104(2): 533–550. https://doi.org/10.1094/PDIS-03-19-0545-RE

Luo, Y., Niederholzer, F. J. A., Felts, D. G., Puckett, R. D., and Michailides, T. J. 2020. Inoculum quantification of canker-causing pathogens in prune and walnut orchards using real-time PCR. *Journal of Applied Microbiology* 129

Ma, Z., Michailides, T.J., 2005. Advances in understanding molecular mechanisms of fungicide resistance and molecular detection of resistant genotypes in phytopathogenic fungi. *Crop Protection* 24(10): 853-863.

Marincowitz, S., Crous, P. W., Groenewald, J. Z., Wingfield, M. J. 2008. Microfungi occurring on the Proteaceae in the fynbos. *CBS Biodiversity Series* 7: 1–166. CBS Fungal Biodiversity Centre, Utrecht, Netherlands.

McDonald, V., Eskalen, A. 2011. Botryosphaeriaceae species associated with avocado branch cankers in California. *Plant Disease* 95:1465-1473.

McDonald, V., Lynch, S., Eskalen, A. 2009. First report of *Neofusicoccum australe*, *N. luteum*, and *N. parvum* associated with avocado branch canker in California. *Plant Disease* 93:967.

Michailides, T.J., and Morgan, D.P. 1993. Spore release by *Botryosphaeria dothidea* in pistachio orchards and disease control by altering the trajectory angle of sprinklers. *Phytopathology* 83:145-152.

Michailides, T.J., and Morgan, D.P. 2004. Panicle and shoot blight of pistachio: A major threat to the California pistachio industry. APS net Feature Story January.

Michailides, T.J., David, P. M., Daniel, G. F., Janine, H., Ryan, P. P., Luo, Y., Mohamed, T. N., Michael, L., Anderson, K., Richard, B., Elizabeth, F., William, C. 2013. Managing *Botryosphaeria*/ *Phomopsis* canker and blight and Anthracnose of walnut in California. Walnut Research Reports. https://ucanr.edu/sites/CEStanislausCo/files/299359.pdf

Michailides, T.J., and Morgan, D.P. 2016. Association of *Botryosphaeria* panicle and shoot blight of pistachio with injuries of fruit caused by Hemiptera insects and birds. *Plant Disease* 100:1405-1413

Morales, A., Latorre, B., Eduardo, P., Besoain, X. 2012. Botryosphaeriaceae species affecting table grape vineyards in Chile and cultivar susceptibility. *Ciencia e investigación agraria*. 39. 445-458. 10.4067/S0718-16202012000300005

Moral, J., Morgan, D. 2019a. Management of *Botryosphaeria* canker and blight diseases of temperate zone nut crops. *Crop Protection*. 104927. 10.1016/j.cropro.2019.104927

Moral, J., Morgan, D., Trapero, A., Michailides, T. J. 2019b. Ecology and Epidemiology of Diseases of Nut Crops and Olives Caused by Botryosphaeriaceae Fungi in California and Spain. *Plant Disease* 103 :1809-1827

Morales-Cruz, A., Amrine, K. C. H., Blanco-Ulate, B., Lawrence, D. P., Travadon, R., Rolshausen, P. E., Baumgartner, K., and Cantu, D. 2015. Distinctive expansion of gene families associated with plant cell wall degradation, secondary metabolism, and nutrient uptake in the genomes of grapevine trunk pathogens. *Bmc Genomics* 16.

Muñóz, M. V. 2017. Nueces: Chile la mayor tasa de crecimiento productivo medio annual. Oficina de Estudios y Politicas Agrarias. https://www.odepa.gob.cl

Nei, M. and Kumar, S. 2000. Molecular Evolution and Phylogenetics. Oxford University Press, New York.

Palma, M.A., Piontelli, E. 2000. Notas Micológicas III. *Diaporthe actinidae* Sommer and Beraha asociado a plantas de kiwi con muerte regresiva en la V Región- Chile. *Boletín Micológico* (Chile) 15:79-83

Palma, M. A., Merello, C., San Martín, J. 2006. Determinación de *Botryosphaeria obtusa* (Schwein) Shoemaker / *Diplodia pinea* complex ocasionando cancrosis en níspero (*Eriobotrya japonica* L.) en Quillota, V Región de Chile. In: Resúmenes XVI Congreso Sociedad Chilena de Fitopatología. 14-17 Noviembre de 2006. La Serena, Chile. http:// www.fitopatologiachile.cl

Pitt, W. Sosnowski, M., Huang, R., Qui, Y., Steel, C., Savocchia, S. 2012. Evaluation of Fungicides for the Management of *Botryosphaeria* Canker of Grapevines. *Plant Disease* 96: 1303-1308. 10.1094/PDIS-11-11-0998-RE

Rolshausen, P. E., Úrbez-Torres, J. R., Rooney-Latham, S., Eskalen, A., Smith, R., Gubler W. D. 2010. Evaluation of pruning wound susceptibility and protection against fungi associated with grapevine trunk diseases. *American Journal of Enology and Viticulture* 61:113-119.

Rumbos, I. 2007. Twig and branch dieback of walnut trees induced by *Botryosphaeria ribis*. *Plant Pathology* 36: 602 - 605. 10.1111/j.1365-3059.1987.tb02281.x.

Sakalidis, M., Slippers, B., Wingfield, B., Hardy, G., Burgess, T. 2013. The challenge of understanding the origin, pathways and extent of fungal invasions: Global populations of the *Neofusicoccum parvum-N. ribis* species complex. *Diversity and Distributions* 19: 873-883. 10.1111/ddi.12030

Servicio Agrícola y Ganadero: SAG, Chile. 2021. http://www.sag.cl/ambitos-de-accion/plaguicidas-y-fertilizantes

Slippers, B., Wingfield, M. 2007. Botryosphaeriaceae as endophytes and latent pathogens of woody plants: diversity, ecology and impact. *Fungal Biology* Reviews 21: 90-106. 10.1016/j.fbr.2007.06.002.

Smit, W. A., Viljoen, C. D., Wingfield, B. D., Wingfield, M. J., Calitz, F. J. 1996 – A new canker disease of apple, pear, and plum rootstocks caused by *Diaporthe ambigua* in South Africa. *Plant Disease* 80: 1331–1335.

Sohrabi, M., Mohammadi, H., León, M., Armengol, J., and Banihashemi, Z. 2020. Fungal pathogens associated with branch and trunk cankers of nut crops in Iran. *European Journal Plant Pathology* 157:327-351

Tennakoon, K. M.S., Ridgway, H. J., Jaspers, M. J., Jones, E. E. 2017. Botryosphaeriaceae species associated with blueberry dieback and sources of primary inoculum in propagation nurseries in New Zealand. *European Journal Plant Pathology*. https://doi.org/10.1007/s10658-017-1283-9

Udayanga, D., Castlebury, L.A., Rossman, A.Y., Chukeatirote, E., Hyde, K.D. 2014b. Insights into the genus *Diaporthe*: phylogenetic species delimitation in the *D. eres* species complex. *Fungal Diversity* 67:203–229

Udayanga, D., Castlebury, L.A., Rossman, A.Y., Chukeatirote, E., Hyde, K.D. 2015. The *Diaporthe sojae* species complex: phylogenetic re-assessment of pathogens associated with soybean, cucurbits and other field crops. *Fungal Biology* 119:383–407

Uecker, F.A. 1988. A world list of *Phomopsis* names with notes on nomenclature, morphology and biology. *Mycological Memoirs* 13:1–231

Úrbez-Torres, J.R., Gubler, W.D. 2009. Pathogenicity of Botryosphaeriaceae species isolated from grapevine cankers in California. *Plant Disease* 93:584-592.

Valencia, D., Torres, C., Camps, R., López, E., Celis-Diez, J., Besoain, X. 2015. Dissemination of Botryosphaeriaceae conidia in vineyards in the semiarid Mediterranean climate of the Valparaíso Region of Chile. *Phytopathologia Mediterranea* 54: 394-402. 10.14601/Phytopathol_Mediterr-16055.

Valencia, A. L., Gil, P. M., Latorre, B. A., Rosales, I. M. 2019. Characterization and Pathogenicity of Botryosphaeriaceae Species Obtained from Avocado Trees with Branch Canker and Dieback and from Avocado Fruit with Stem End Rot in Chile. *Plant disease* 103(5): 996–1005. https://doi.org/10.1094/PDIS-07-18-1131-RE

Whitelaw-Weckert M. A., Rahman, L., Appleby, L. M., Hall, A., Clark, A. C., Waite, H. Hardie, W. J. 2013. Co-infection by Botryosphaeriaceae and spp. fungi during propagation causes decline of young, grafted grapevines. *Plant Pathology* 62(6):1226–1237. https://doi.org/10.1111/ppa.12059

White, T., Bruns, T., Lee, S., Taylor, J., Innis, M., Gelfand, D., Sninsky, J. 1990. Amplification and Direct Sequencing of Fungal Ribosomal RNA Genes for Phylogenetics.

Zapata, M., Palma, M.A., Aninat, M.J., Piontelli, E. 2020. Polyphasic studies of new species of *Diaporthe* from native forest in Chile, with descriptions of *Diaporthe araucanorum* sp. nov., *Diaporthe foikelawen* sp. nov. and *Diaporthe patagonica* sp. nov. *International journal of systematic and evolutionary microbiology* 70(5): 3379–3390. https://doi.org/10.1099/ijsem.0.004183

https://www.nutfruit.org/files/tech/1524481168_INC_Statistical_Yearbook_2017-2018.pdf

https://www.redagricola.com/cl/los-desafios-para-el-nogal-en-chile/

Table

Table 1.1 Botryosphaeriaceae and Diaportheaceae reference samples with accession numbers from the NCBI database were used for phylogenetic analyses.

Identity	Collection code	Host	Collector	Country of Origin	ITS	EF	B-TUB
Botryosphaeria dothidea	CMW 8000	*Prunus* sp.	B. Slippers	Switzerland	AY236949	AY236898	AY236927
Botryosphaeria dothidea	CMW 7780	*Fraxinus excelsior*	B. Slippers	Switzerland	AY236947	AY236896	AY236925
Diplodia africana	RGM 2718	*Araucaria araucana*	D. Rupaillan	Chile	MN046380	-	-
Diplodia africana	CBS 120835	-	Vu et al. 2017	South Africa	MH863094	-	-
Diplodia mutila	CBS 112553	*Vitis vinifera*	A.J.L. Phillips	Portugal, Montemor-o-Novo	AY259093	AY573219	DQ458850
Diplodia mutila	CBS 230.30	*Phoenix dactylifera*	Alves, A., Correia, A. and Phillips, A.J.L.	USA	DQ458886	DQ458869	DQ458849
Diplodia mutila	4D33	*Persea americana*	-	USA, California, Ventura.	KF778789	KF778979	KF778884
Diplodia mutila	PALUC1M	*Persea americana*	Ana L. Valencia	Chile	MF568683	-	-
Diplodia mutila	DMnog4	*Juglans regia*	Gonzalo Diaz and Enrique Ferrada	Chile	MG386824	-	-
Diplodia mutila	Mz-F22	*Malus domestica*	Diaz et al. 2016	Chile	MG450386	-	-
Diplodia mutila	Sample 301	*Juglans regia*	I. Jiménez Luna	Chile	MW412902	MW574125	MW596891
Diplodia pinea	CMW 39341	*Cedrus deodara*	M. Zlatkovic	Montenegro	KF574998	KF575028	KF575094
Diplodia pinea	CMW 39338	*Cedrus atlantica*	M. Zlatkovic	Serbia	KF574999	KF575029	KF575095
Diplodia sapinea	CMW 190	*Pinus resinosa*	M.A. Palmer	USA	KF766159	AY624251	AY624256
Diplodia seriata	CBS 112555	*Vitis vinifera*	A. J. L. Phillips	Portugal	AY259094	AY573220	DQ458856
Diplodia seriata	CBS 119049	*Vitis* sp.	A. J. L. Phillips	Italy	DQ458889	DQ458874	DQ458857
Diplodia seriata	PALUC14M	*Persea americana*	Ana L. Valencia	Chile	MF578223	-	-
Diplodia seriata	KJ 93.56	*Vitis vinifera*	Jacobs and Rehner	Chile	AF027759	-	-
Diplodia seriata	Mz-F1	*Malus domestica*	Diaz et al. 2016	Chile	KU942427	-	-
Diplodia seriata	Sample 105	*Juglans regia*	I. Jiménez Luna	Chile	MW412901	MW574124	MW596890
Diplodia scrobiculata	CBS 109944	*Pinus greggii*	M.J. Wingfield	Mexico	DQ458899	DQ458884	DQ458867
Diplodia scrobiculata	CBS 113423	*Pinus greggii*	M.J. Wingfield	Mexico	DQ458900	DQ458885	DQ458868
Dothiorella iberica	CBS 115041	*Quercus ilex*	J. Luque	Spain	AY573202	AY573222	EU673096
Dothiorella iberica	CBS 113188	*Quercus suber*	M. E. Sanchez	Spain, Andalucia	AY573198	EU673278	EU673097
Dothiorella iberica	PALUC3M	*Persea americana*	Ana L. Valencia	Chile	MF578225	-	-
Dothiorella sarmentorum	CBS 115038	*Malus pumila*	A. J. L. Phillips	The Netherlands	AY573206	AY573223	EU673101
Lasiodiplodia citricola	6I34	*Juglans regia*	Chen et al. 2014	Stanislaus, CA	KF778809	KF778999	KF778904
Lasiodiplodia citricola	CBS 124707	*Citrus sp.*	J. Abdollahzadeh and A. Javadi	Iran	GU945354	GU945340	KU887505

Lasiodiplodia citricola	IRNKB3	*Juglans regia*	H. Mohammadi	Eghlid, Fars province	MN634040	MN633994	MN633442
Lasiodiplodia pseudotheobromae	CBS 116459	*Gmelina arborea*	J. Carranza-Velasquez	Costa Rica	EF622077	EF622057	EU673111
Lasiodiplodia theobromae	CBS 164.96	Fruit along coral reef coast	-	Papua New Guinea	AY640258	AY640255	EU673110
Lasiodiplodia theobromae	PALUC449F	*Persea americana*	Ana L. Valencia	Chile	MF578754	-	-
Neofusicoccum arbuti	B03-07	*Blueberry* cv. Aurora	J.G. Espinoza	Chile	EU856061	-	-
Neofusicoccum australe	CMW 6837	*Acacia* sp.	Slippers et al. 2004b	Australia	AY339262	-	-
Neofusicoccum australe	CAP258	*Olea europaea*	Lazzizera et al. 2008	Italy	EF638778	-	-
Neofusicoccum australe	PALUC439F	*Persea americana*	Ana L. Valencia	Chile	MF578755	-	-
Neofusicoccum australe	B1-05	Blueberry cv. *Duke*	E.X. Briceno	Chile	EU856059	-	-
Neofusicoccum australe	vid-1559	*Vitis vinifera*	C. Torres	Chile	JX290091	-	-
Neofusicoccum mediterraneum	6I29	*Juglans regia*	Michailides T.	Stanislaus, CA	KF778849	KF779039	KF778944
Neofusicoccum nonquaesitum	UCR2733	*Persea americana*	Carillo et al. 2006	USA	KT965281		-
Neofusicoccum nonquaesitum	PALUC4M	*Persea americana*	Ana L. Valencia	Chile	MF578228		-
Neofusicoccum nonquaesitum	CABI IMI-500168	*Vaccinium corymbosum*	Perez et al. 2012	Chile	JX217819	-	-
Neofusicoccum nonquaesitum	4L78	*Juglans regia*	Chen et al. 2014	Sutter, CA	KF778851	KF779041	KF778946
Neofusicoccum nonquaesitum	PD90	*Prunus dulcis*	T. J. Michailides	Butte, CA	GU251157	GU251289	GU251817
Neofusicoccum parvum	CBS 110301	*Vitis vinifera*	-	Portugal	AY259098	AY573221	EU673095
Neofusicoccum parvum	CMW9080	*Populus nigra*	Slippers et al. 2004a	New Zealand	AY236942	-	-
Neofusicoccum parvum	CMW 9081	*Populus nigra*	G.J. Samuels	New Zealand, TE Puke	AY236943	AY236888	AY236917
Neofusicoccum parvum	PALUC16M	*Persea americana*	Ana L. Valencia	Chile	MF578229	-	-
Neofusicoccum parvum	B1-06	*Blueberry* cv. Misty	E.X. Briceno	Chile	EU856063	-	-
Neofusicoccum parvum	Sample 172	*Juglans regia*	I. Jiménez Luna	Chile	MW412903	MW574126	MW596892
Neofusicoccum vitifusiforme	5H02	*Juglans regia*	Chen et al. 2014	Fresno, CA	KF778868	KF779058	KF778963
Neofusicoccum vitifusiforme	CBS 110881	*Vitis vinifera*	J. M. van Niekerk	South Africa	AY343383	AY343343	KX465061
Neoscytalidium dimidiatum	CBS 499.66	*Mangifera indica*	Lopez-Moral et al. 2019	Mali	FM211432	EU144063	FM211167
Diaportheaceae							
Diaporthe ambigua	CBS 114015	*Pyrus communis*	S. Denman	South Africa	KC343010	-	-
Diaporthe ambigua	6-KF	*Actinidia deliciosa*	Diaz et al. 2014	Chile	KJ210025	-	-
Diaporthe ambigua	5.5.4r1(2)	*Vaccinium* sp.	Elfar et al. 2013	Chile	KC143171	-	-
Diaporthe ampelina	CBS 111888	*Vitis vinifera*	J.D. Cucuzza	USA	KC343016	KC343742	KC343984
Diaporthe araucanorum	RGM 2472	*Araucaria araucana*	P. Pulgar	Chile	MN509709	-	-

Diaporthe asheicola	CBS 136968	*Vaccinium ashei*	A. Schilder	Chile	KJ160563	KJ160595	KJ160519
Diaporthe asheicola	CBS 136967	*Vaccinium ashei*	A. Schilder	Chile	KJ160562	KJ160594	KJ160518
Diaporthe australafricana	CBS 111886	*Vitis vinifera*	R.W.A. Schepers	Australia	KC343038	KC343764	KC344006
Diaporthe australafricana	CBS 113487	*Vitis vinifera*	L. Mostert	South Africa	KC343039	KC343765	KC344007
Diaporthe australafricana	16-KF	*Actinidia deliciosa*	Gonzalo Diaz	Chile	KX999702	-	-
Diaporthe australafricana	Pho73-07	*Vaccinium* sp.	Elfar et al. 2013	Chile	KC143190	-	-
Diaporthe australafricana	15.2.2(4)	*Vaccinium* sp.	Elfar et al. 2013	Chile	KC143175	-	-
Diaporthe australafricana	Sample 302	*Juglans regia*	I. Jiménez Luna	Chile	MW407063	MW574121	MW574123
Diaporthe beckhausii	CBS 138.27	*Viburnum* sp.	L.E. Wehmeyer	-	KC343041	KC343767	KC344009
Diaporthe chamaeropis	CBS 454.81	*Chamaerops humilis*	H.A van der Aa	Greece	KC343048	KC343774	KC344016
Diaporthe chamaeropis	CBS 753.70	*Spartium junceum*	J.A. von Arx	Croatia	KC343049	KC343775	KC344017
Diaporthe cynaroidis	CBS 122676	*Protea cynaroidis*	S. Marincowitz	South Africa	KC343058	KC343784	KC344026
Diaporthe cynaroidis	Sample 102	*Juglans regia*	I. Jiménez Luna	Chile	MW407062	MW574120	MW574122
Diaporthe eres	CBS 101742	*Fraxinus* sp.	G.J.M. Verkley	Netherlands	KC343073	KC343799	KC344041
Diaporthe eres	CPC 16510	*Vaccinium corymbosum*	Lombard et al. 2014	Chile	KJ160572	-	-
Diaporthe foeniculina	CBS 117166	*Aspalathus linearis*	J.C. Van Rensburg, et al. 2006	South Africa	DQ286286	-	-
Diaporthe foikelawen	RGM 2539	*Drimys winteri*	M. Espinoza	Chile	MN509713	-	-
Diaporthe neotheicola	CBS 123208	*Foeniculum vulgare*	A.J.L. Phillips	Portugal	EU814480	GQ250315	JX275464
Diaporthe neotheicola	6I30	*Juglans regia*	Chen et al. 2014	Stanislaus, CA	KF778871	KF779061	KF778966
Diaporthe neotheicola	3.4.4r1(1)	*Vaccinium* sp.	Elfar et al. 2013	Chile	KC143192	-	-
Diaporthe neotheicola	ColPat-445	*Juglans regia* cv. Tulare	C. Agusti-Brisach and A. Trapero	Benavides, Badajoz, Spain	MK447993	MK490932	MK522106
Diaporthe neotheicola	ColPat-448	*Juglans regia* cv. Serr	C. Agusti-Brisach and A. Trapero	Benavides, Badajoz, Spain	MK447994	MK490939	MK522107
Diaporthe neotheicola	ColPat-450	*Juglans regia* cv. Vina	C. Agusti-Brisach and A. Trapero	Cordoba, Spain	MK447996	MK490934	MK522109
Diaporthe neotheicola	ColPat-532	*Juglans regia* cv. Chandler	C. Agusti-Brisach and A. Trapero	Alcala del Rio, Sevilla, Spain	MK447998	MK490936	MK522111
Diaporthe neotheicola	ColPat-551	*Juglans regia* cv. Hartley	C. Agusti-Brisach and A. Trapero	Badajoz, Spain	MK448000	MK490940	MK522112
Diaporthe nobilis	CBS 200.39	*Laurus nobilis*	Kotthoff	Germany	KC343151	KC343877	KC344119
Diaporthe novem	CBS 127271	*Glycine max*	T. Duvnjak	Croatia	KC343157	-	-
Diaporthe novem	1-KF	*Actinidia deliciosa*	Gonzalo Diaz	Chile	KJ210020	-	-
Diaporthe passiflorae	CPC 19183	*Passiflora edulis*	P.W. Crous	South America	JX069860	-	-
Diaporthe passiflorae	15.3.1r1	*Vaccinium* sp.	Elfar et al. 2013	Chile	KC143196	-	-

Diaporthe rudis	CBS 114011	*Vitis vinifera*	-	Portugal	KC343235	KC343961	KC344203
Diaporthe rudis	CBS 113201	*Vitis vinifera*	-	Portugal	KC343234	KC343960	KC344202
Diaporthe rhusicola	CBS 129528	*Rhus pendulina*	P. W. Crous	Western Cape, South Africa	JF951146	KC843100	KC843205
Diaporthe rhusicola	6I14	*Prunus dulcis*	Chen et al. 2014	Kings, CA	KF778872	KF779062	KF778967
Diaporthe rhusicola	6I31	*Juglans regia*	Chen et al. 2014	Stanislaus, CA	KF778874	KF779064	KF778969
Diaporthe rhusicola	ColPat-444	*Juglans regia* cv. Tulare	C. Agusti-Brisach and A. Trapero	Benavides, Badajoz, Spain	MK447992	MK490931	MK522105
Diaporthe sterilis	CBS 136969	*Vaccinium corymbosum*	G. Polizzi	Italy	KJ160579	KJ160611	KJ160528
Diaporthe sterilis	CBS 136970	*Vaccinium corymbosum*	G. Polizzi	Italy	KJ160580	KJ160612	KJ160529
Diaporthe toxica	CBS 534.93	*Lupinus angustifolius*	J.B. Nunn	Western Australia	KC343220	KC343946	kC344188
Diaporthe toxica	CBS 546.93	*Lupinus* sp.	P.M. Williamson	Western Australia	KC343222	KC343948	KC344190
Diaporthe vaccinia	CBS 160.32	*Vaccinium macrocarpon*	C.L. Shear	USA	KC343228	KC343954	KC344196
Diaporthe woodii	CBS 449.82	*Lupinus* sp.	H.A. van der Aa	Netherlands	KC343240	KC343966	KC344208
Phomopsis amygdali	ColPat-533	*Juglans regia* cv. Chandler	C. Agusti-Brisach and A. Trapero	Alcala del Rio, Sevilla, Spain	MK447999	MK490937	MK522117
Phomopsis amygdali	CBS 126679	*Prunus dulcis*	E. Diogo	Portugal	KC343022	KC343748	KC343990
Phomopsis amygdali	CBS 115620	*Prunus persica*	W. Uddin	Georgia, USA	KC343020	KC343746	KC343988
Phomopsis controversa	CBS 100170	*Fraxinus excelsior*	H.A. van der Aa	Netherlands	KC343230	KC343956	KC344198
Phomopsis heveae	CBS 852.97	*Hevea brasiliensis*	D.S. Attili	Brazil	KC343116	KC343842	KC344084

Figures

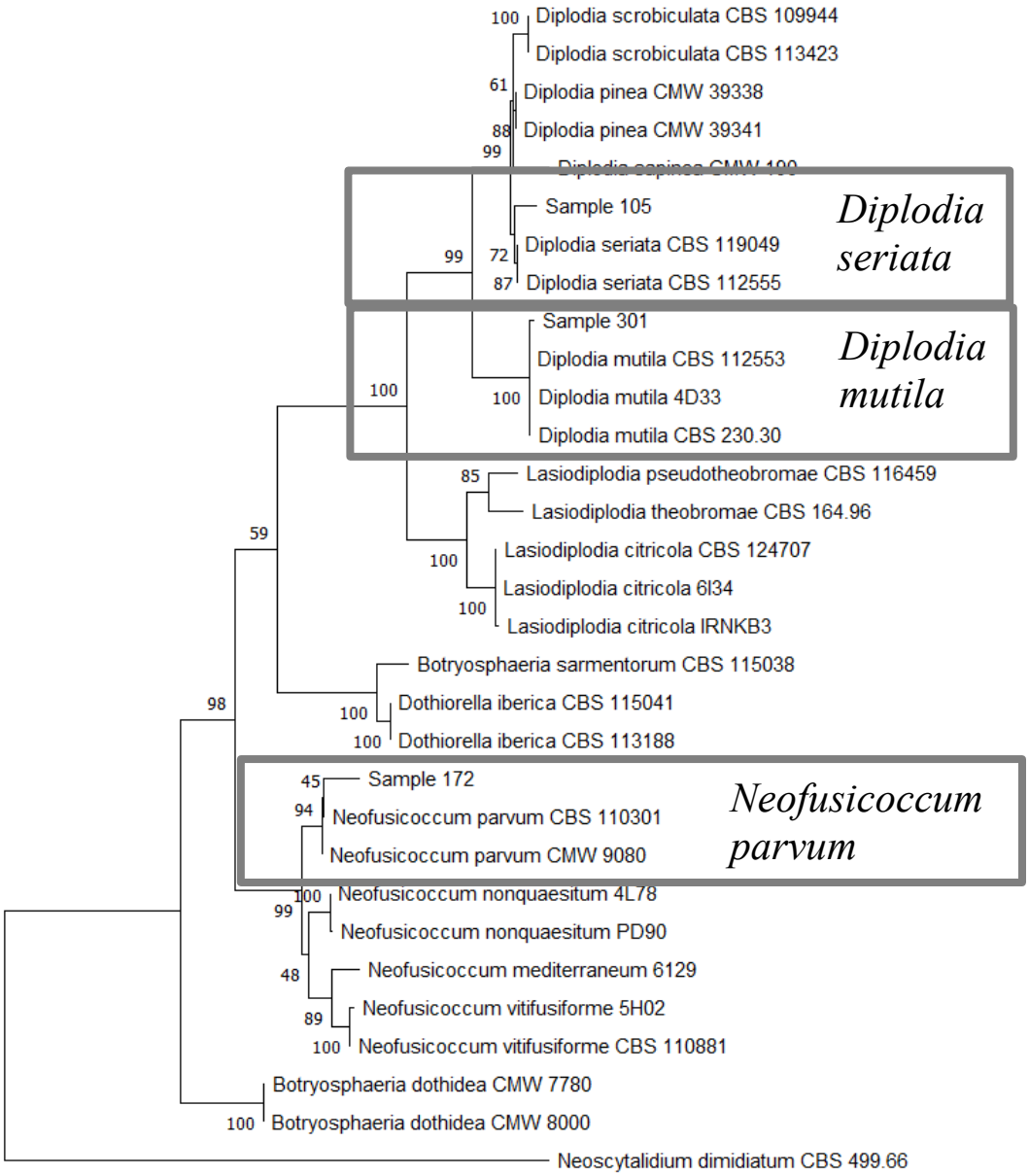


Figure 1.1 Phylogenetic tree reconstructed by maximum likelihood analysis from the concatenated sequences of the nuclear ribosomal internal transcribed spacer, translation elongation factor and beta-tubulin for 3 Botryosphaeriaceae taxa isolated from three walnut orchards in Chile and 28 *Botryosphaeria* reference sequences retrieved from the GenBank database. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Numbers on branches are bootstrap support values.

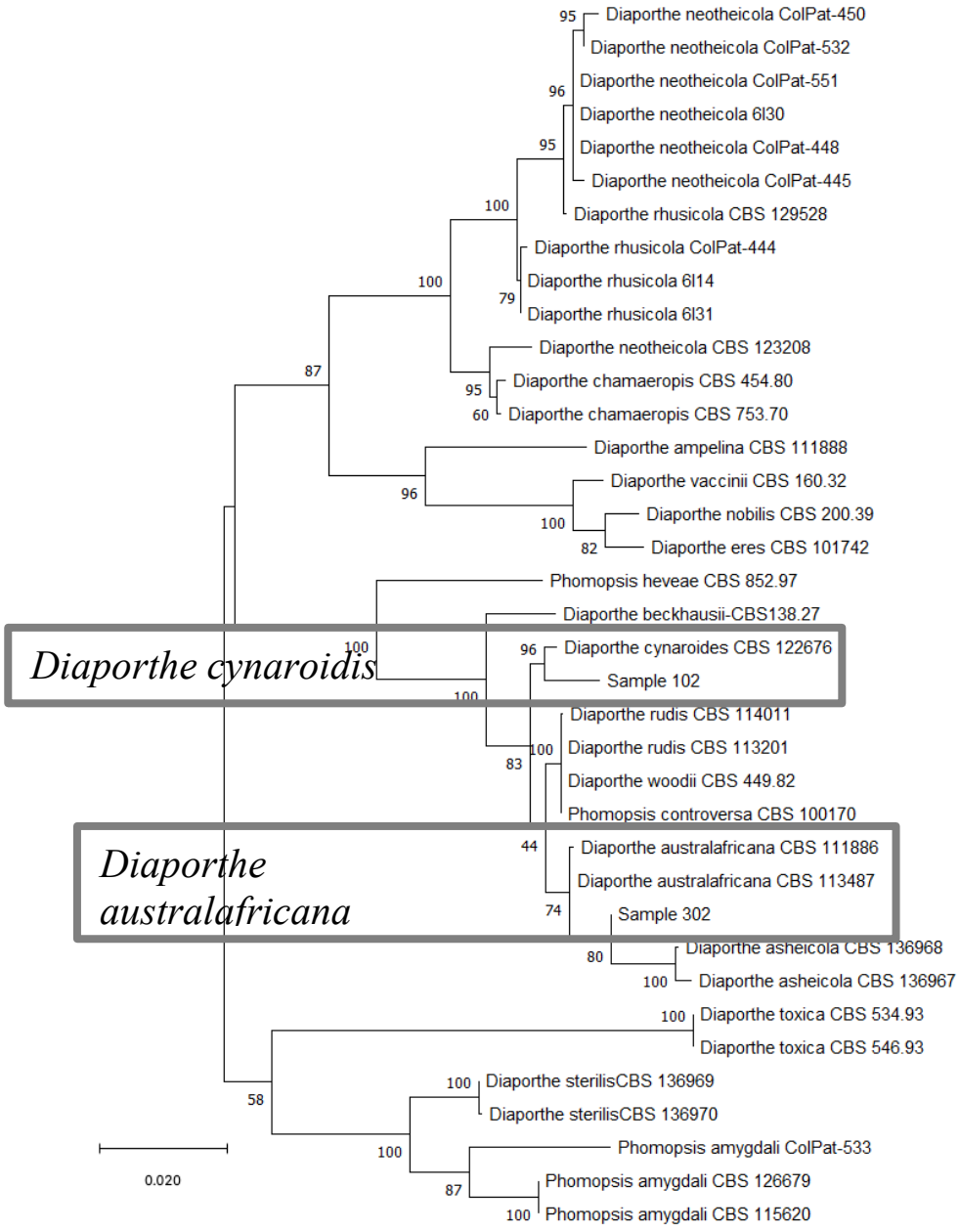
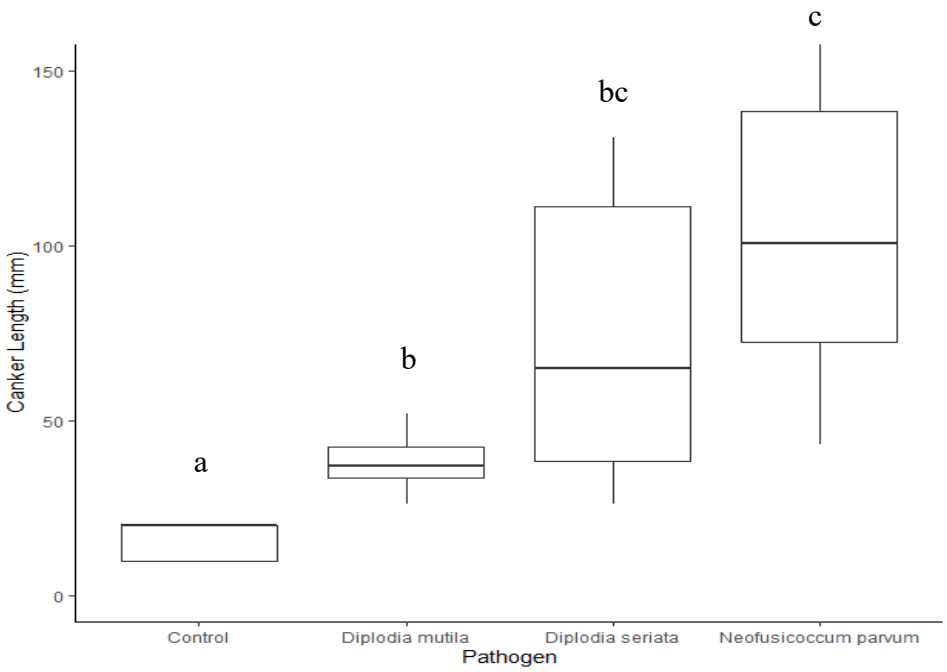


Figure 1.2. Phylogenetic tree reconstructed by maximum likelihood analysis from the concatenated sequences of the nuclear ribosomal internal transcribed spacer, translation elongation factor and beta-tubulin for two Diaportheaceae taxa recovered from two walnut orchards in Chile, and 35 *Diaporthe* reference sequences retrieved from the GenBank database. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Numbers on branches are bootstrap support values.

A



B

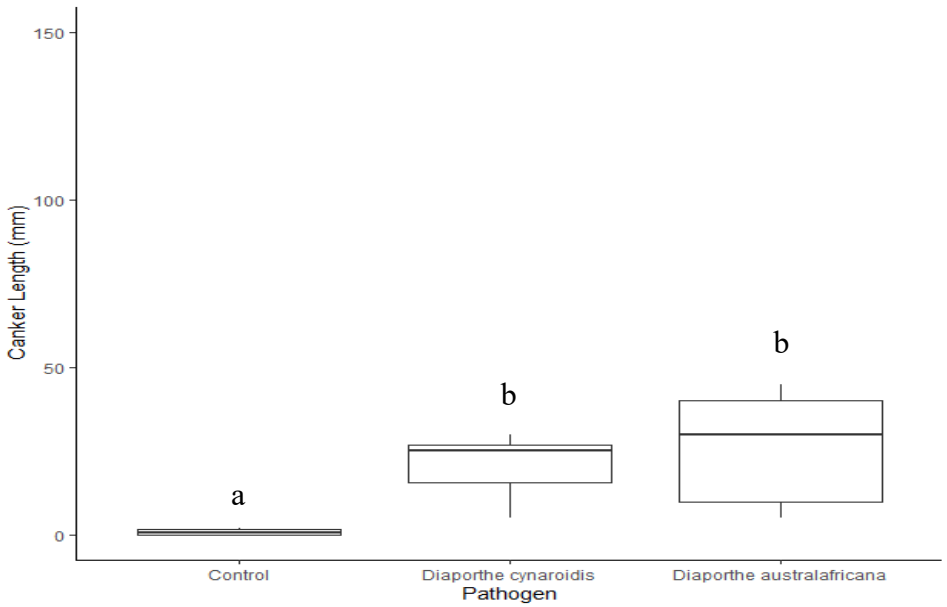


Figure 1.3. Average canker length (mm) after 12 weeks caused by three Botryosphaeriaceae species (**A**), and two *Diaporthe* species (**B**) on walnut stems inoculated with mycelial plugs ($P < 0.05$). Similar letters show no statistical significance between treatments.

Supplemental Figures

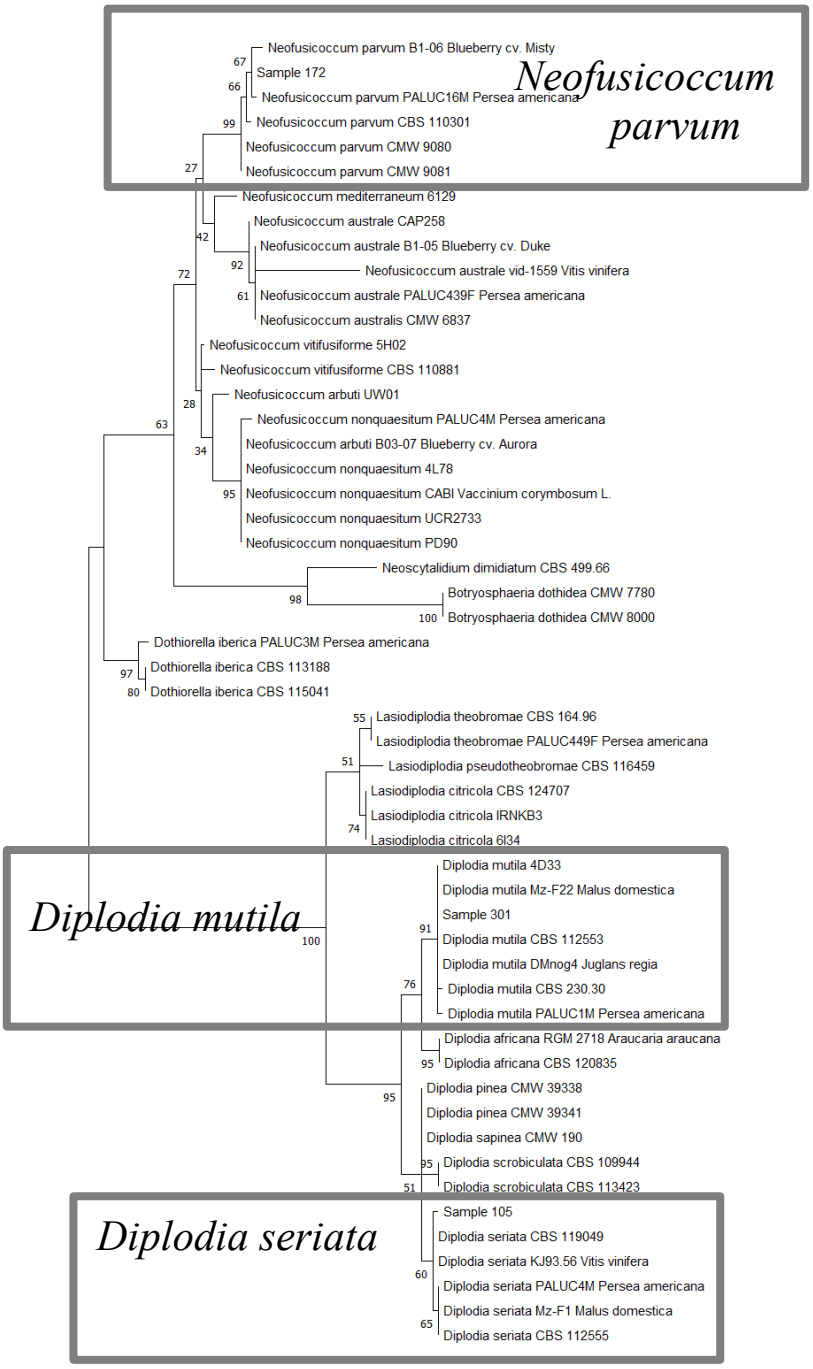


Figure S1.1. Phylogenetic tree reconstructed by maximum likelihood analysis from the concatenated sequences of the nuclear ribosomal internal transcribed spacer for three Botryosphaeriaceae taxa isolated from three walnut orchards in Chile and 50 *Botryosphaeria* reference sequences retrieved from the GenBank database. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Numbers on branches are bootstrap support values.

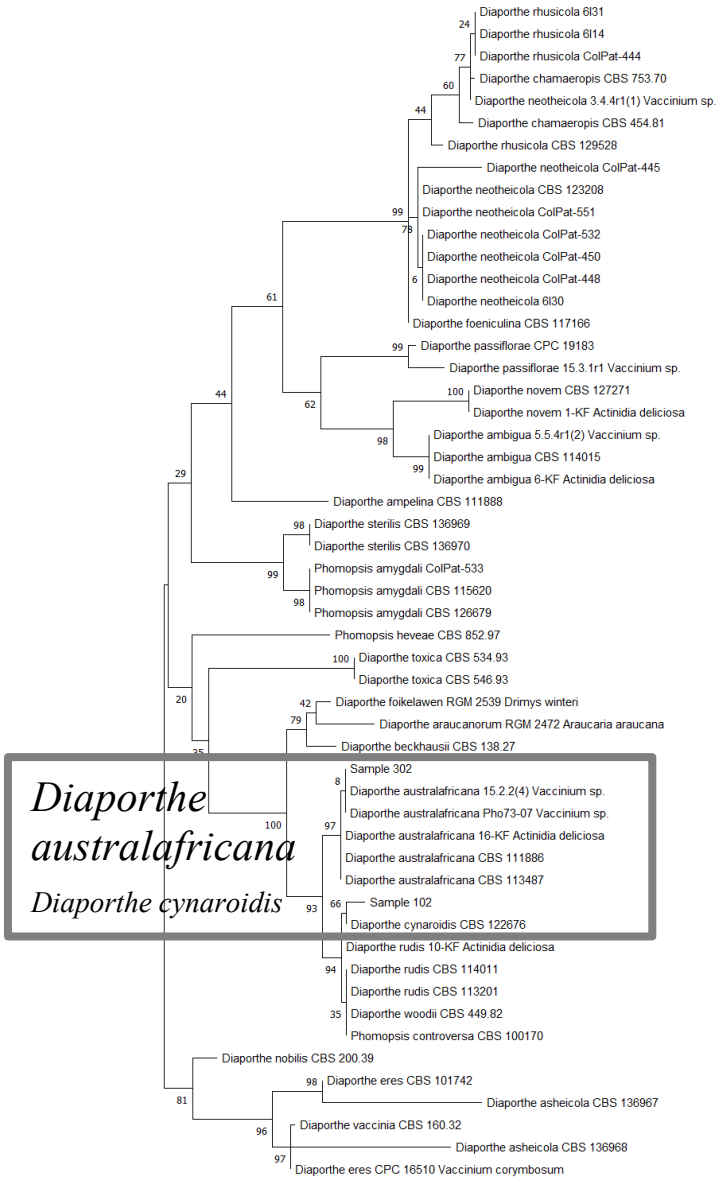


Figure S1.2. Phylogenetic tree reconstructed by maximum likelihood analysis from the concatenated sequences of the nuclear ribosomal internal transcribed spacer for two Diaportheaceae taxa recovered from two walnut orchards in Chile, and 51 *Diaporthe* reference sequences retrieved from the GenBank database. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Numbers on branches are bootstrap support values.

Chapter II

Qualitative Spore Trapping of Wood Canker Pathogens in California Almond and Walnut Orchards

Abstract

Fungal wood diseases are major limiting factors to woody perennial crop productivity and longevity. The goal of the study was to qualitatively assess fungal airborne inoculum causing wood diseases in commercial orchards and determine if its profile is influenced by tree host, age, weather and location. We selected 9 trees within three almond orchards and one walnut orchard with different characteristics. Fungal inoculum was captured weekly using a passive spore trapping device during a 30 week-period in the dormant season (November-April), for two consecutive years per orchard. Spore trapping was complemented with collection of symptomatic wood samples from orchards showing signs of dieback and necrosis. We identified all taxa using a culture-dependent approach coupled with Sanger sequencing and phylogenetic analyses. Our results showed that Botryosphaeriaceae and Diaportheaceae were the dominant airborne groups but while those were also found in affected trees, the insect-vectored *Ceratocystis* was the main pathogen recovered from almond wood samples. Phylogenetic analyses identified eight putative species including five genera in the Botryosphaeriaceae family (*Diplodia corticola, Diplodia mutila*, *Diplodia seriata*, *Dothiorella iberica/D. sarmentorum*, *Neofusicoccum parvum*, *Neofusicoccum mediterraneum*, *Neoscytalidium dimidiatum*, and *Spencermartinsia viticola*) and three species in one genus in the Diaportheaceae family

(*Diaporthe ampelina*, *Diaporthe eres* and *Diaporthe chamaeropis*). We proposed that host type, orchard age, wet events, and alternate hosts at the periphery of orchards were factors that could affect airborne pathogen profile. Inoculum within orchards was composed of endemic taxa characterized by frequent and repeated trapping events from the same trees and immigrant taxa characterized by rare and random trapping events. We discuss the limitations and benefits of our approach to developing guidelines and prediction tools for fungal canker diseases in California orchards.

Introduction

In 2018, the US ranked first and second in almond (*Prunus dulcis*) and walnut (*Juglans regia*) production worldwide, respectively (USDA-NASS 2019). California accounts for over 99% of the nation's production for those nut crops, with about 630 thousand planted hectares (480,000 ha of almond and 150,000 ha of walnut) and an estimated combined value of $7.4 billion ($6.1 billion for almond and $1.3 billion for walnut) (CDFA 2020). However, as these industries look to expand to meet the global demand, several factors are hindering those prospects. Among them, fungal wood diseases are impacting the longevity and productivity of perennial cropping systems. For example, they were responsible for a yield reduction ranging from 30-60% and 40-100% in California vineyards (Munkvold et al. 1994) and pistachio orchards (Moral et al. 2019b), respectively. In addition, wood diseases caused mortality of about 5% of walnut trees in nursery (Chen et al. 2013a) and 25% of almond trees in young orchards (Chen et al. 2013b).

Band canker and *Ceratocystis* canker are some of the common wood diseases in almond orchards (Teviotdale et al. 1996). Thousand cankers and Botryosphaeria canker are

also important wood diseases of walnut (Leslie et al. 2009). Wood canker results in the loss of physiological functions of the host vascular system in response to fungal infection (Pouzoulet et al. 2014). Cankers are visible externally on the bark of branches or trunks of trees and are induced following the death of the plant vascular cambium (Pearce 1996; Shigo and Marx 1977). When a canker encircles a major branch or a tree trunk, it girdles the wood entirely causing branch dieback or decline of the entire tree (Inderbitzin et al. 2010; Olmo et al. 2016). Additional symptoms in the tree canopy can also include limb and twig dieback, canopy wilting and thinning, shoot blight, leaf chlorosis and leaf spot, while internal symptoms in the wood include wedge-shaped dark patches, ring of discoloration and brown to dark vascular streaking (Holland et al. 2020; Lawrence et al. 2015; León et al. 2020; López-Moral et al. 2020a). In almond, infection by canker pathogens is often accompanied externally by amber-colored balls of gum on the bark (Gramaje et al. 2012; Holland et al. 2020).

Wood diseases are caused by a complex of fungal pathogens unrelated taxonomically with broad host and geographical ranges. Among those, taxa in the families Botryosphaeriaceae and Diaportheaceae have been recognized as major causal agents of cankers in almond and walnut production systems globally (Abdollahzadeh et al. 2014; Adaskaveg et al. 1999; Diogo et al. 2010; Doll et al. 2015; English et al. 1966; Gramaje et al. 2012; Inderbitzin et al. 2010; Jimenez Luna et al. 2020; Lawrence et al. 2015; León et al. 2020; López-Moral et al. 2020a; Nouri et al. 2018; Olmo et al. 2016; Sohrabi et al. 2020; Wunderlich et al. 2012). Several additional fungal genera have also been associated with the complex of vascular diseases in almond and walnut including *Ceratocystis,*

Collophora, Cytospora, Eutypa, Geosmithia, and *Phaeoacremonium* (Gramaje et al. 2012; Holland et al. 2018; Holland et al. 2020; Kolarik et al. 2011; Lawrence et al. 2018; Luo et al. 2020; Sohrabi et al. 2020; Trouillas and Gubler 2010). Many of those pathogens are airborne and infect open wounds of the plant host (Luo et al. 2019). Spore-trapping studies in vineyards and orchards have clearly shown that rain, sprinkler irrigation, or high relative humidity for some taxa, can trigger spore release (Ahimera et al. 2004; Eskalen et al. 2013; Luo et al. 2020; Pusey 1989; Urbez-Torres et al. 2010; van Niekerk et al. 2010). In contrast, other fungal pathogens are vectored by insects, including the causal agents of Ceratocystis canker in almond and thousand cankers in walnut, and tree infection tracks insect boring behavior (Kolarik et al. 2011; Moller and DeVay 1968).

Canker diseases have become a growing concern among industry stakeholders and are the result of a recent shift in cultural practices (Doll et al. 2013). Tighter tree planting densities to maximize land resources combined with an aggressive nutritional program for faster and higher yields have resulted in larger pruning wounds thereby increasing chances of infection as recently shown in vineyards (Henderson et al. 2021). More frequent indiscriminate hedging cuts to contain canopy volume and architecture while optimizing fruit set could also result in an increased number of possible entry points for airborne pathogens causing canker diseases. Moreover, wounds induced by mechanical shaking of trunks at harvest and natural cracks of the tree bark caused by vigorous tree growth and heavy crop load are common and provide additional routes for wood pathogens to infect trees (Holland et al. 2020; Moral et al. 2019a; Moral et al. 2019b).

The aim of this study was to qualitatively profile the airborne inoculum of canker pathogens in California almond and walnut orchards using a spore trapping method combined with a culturing and multi locus DNA phylogeny approach. We hypothesized that inoculum in orchards is composed of both cosmopolitan and unique pathogens and that inoculum profile is influenced by orchard crop, age, climate and location. The results of this study improve our current knowledge of the epidemiology of canker-causing pathogens in almond and walnut and contribute to the implementation of adapted management strategies.

Methods

Fungal culture and collection.

We selected three almond orchards and one walnut orchard located in Merced County, California. The first almond orchard (Almond #1) was ten-year-old and located within 400 meters of a riparian area and had adjacent vineyard and walnut plantings. Three almond cultivars were planted within this orchard and included 'Nonpareil', 'Carmel', and 'Monterey' grafted to the rootstock 'Bright's Hybrid'. The second orchard (Almond #2) was six-year-old and had a history of canker diseases. It was surrounded by other almond orchards and was planted with cultivars 'Nonpareil,' 'Aldrich,' and 'Monterey' grafted to the rootstock 'Viking.' The last almond orchard (Almond #3) was four-year-old and composed of 'Nonpareil,' 'Carmel,' and 'Aldrich' grafted to 'Bright's Hybrid' rootstock with other almond plantings nearby. The last orchard was a mature fifteen-year-old 'Tulare' walnut orchard grafted on 'Paradox' rootstock and was selected due to its proximity to the 'Almond #3' orchard.

We recorded total precipitation (mm) and number of wet events (precipitation with at least 0.25 mm of rainfall in a single week) weekly using two California Irrigation Management Information System stations (Merced and Denair CIMIS; https://cimis.water.ca.gov/) close to the commercial orchards where the experiments were conducted.

The spore trapping method was adapted from Eskalen et al. (2013) and Urbez-Torres et al. (2010). Spore traps consisted of microscope slides coated on both sides with Petroleum jelly attached to a limb on the north side of the tree at 2 meters height. In each orchard, traps were placed on nine trees within three rows, with three trees per row. Traps were collected weekly and replaced the same day for a total of 30 weeks from November through April. Spore trapping was repeated for two consecutive seasons for each orchard and the trials spanned from 2012 to 2016 for all four orchards.

For slide collection, each slide was placed inside a sterile 50 mL Falcon tube and mailed to the laboratory at UC Riverside where they were further processed. Slides were washed by adding 10 mL of a warm (~30°C) aqueous solution containing 0.5% Tween to the Falcon tube and shaking it for 30 sec. A 200 µl aliquot of this rinsate was cultured in duplicates on PDA (Potato Dextrose Agar, Difco) culture medium amended with three antibiotics (ampicillin, neomycin and tetracycline at 1 mg/L each) to inhibit bacterial growth. For wood samples collection, 23 symptomatic trunk, branch and twig samples were collected from the four orchards and brought back to Kearney Agricultural Research and Extension Center and processed as described by Holland et al. (2020). Briefly, symptomatic wood samples were cut into small wood or bark pieces (~3 mm^3) exposing

the margin of infected and apparently healthy tissues, surface disinfested by submerging in 0.5% sodium hypochlorite for 2 min, then rinsed twice in sterile, deionized water. Pieces were then dried on sterile paper towels and plated (10 pieces per plate) on 90-mm-diameter Petri dishes of PDA amended with lactic acid (APDA). For samples with suspected Ceratocystis canker symptoms, bark pieces exposing the cambium and displaying the margin between cankers and healthy tissues were surface disinfested as described above and incubated in a humid chamber as described previously (Holland et al. 2019). All culture plates were incubated at ambient conditions in the laboratory. After 7 days, fungi were selected based on morphological characters and transferred to new PDA culture medium. Most of the Botryosphaeria-like isolates were identified by DNA sequences whereas only a small subset of *Diaporthe*-like isolates was identified using the same method because too many isolates were recovered from spore traps.

DNA extraction, PCR amplification and DNA sequencing.

DNA was extracted from fungal pure cultures obtained from both slides and isolations using the Qiagen Blood and Tissue Kit (Qiagen, Valencia, California) according to the manufacturer's instructions. PCR amplification was performed in 25 µL reaction volumes on a T100 thermocycler (BioRad, Irvine, California). Each reaction consisted of 17.4 µL sterile H_2O, 2.5 µL PCR buffer, 1 µL dNTPs (10 mM each), 0.5 µL each primer (4 µM), 2 µL $MgCl_2$ (25 mM), and 0.1 µL *Taq* DNA polymerase (5 U/µL), with DNA added at 1 µl (10–20 ng DNA/µl). The Internal Transcribed Spacer (ITS) region was PCR-amplified with the ITS1-ITS4 primer pair (White et al. 1990) and the translation elongation factor 1α region (EF-1α) was amplified with the EF1F-EF2R primer pair (Jacobs et al.

2004). Thermocycler settings consisted of a 2 min initial denaturation step at 94 °C, then 35 cycles of the following three steps: 1 min at 94 °C for strand separation, 1 min at 62 °C for primer annealing and 1 min at 72 °C for amplification; the PCR was completed with a final 3-min extension at 72 °C. Amplicons were run on a 1% agarose gel using gel electrophoresis at 110 Volts for 20 min and stained with Gel Red dye (Biotium Inc., Fremont, California). PCR products were visualized as bands under UV light using a Gel Doc Imager (BioRad, Irvine, California) and amplicons were purified using the QiaQuick PCR Purification Kit (Qiagen, Valencia, California). Forward and reverse reads were generated by Sanger sequencing performed at the UCR Institute of Integrative Genome Biology sequencing core.

Phylogenetic analyses.

Forward and reverse reads of each gene region were edited and combined into a consensus sequence using Sequencher v. 5.0.1 (Gene Codes Corporation, Ann Arbor, MI). Sequences from each data partition (ITS and EF) were concatenated in Geneious v. 6.0.6 (Biomatters Ltd., Auckland, New Zealand) and aligned manually. Sequence alignments and phylogenetic analyses were performed separately for Botryosphaeriaceae and Diaportheaceae owing to their taxonomic divergence. The complete dataset of Botryosphaeriaceae consisted of 33 novel sequences and 23 reference sequences. The Diaportheaceae dataset consisted of four novel sequences and 24 reference sequences. Reference taxa were obtained from fungal culture collections (including the Westerdijk Institute/Central bureau voor Schimmelcultures, CBS-KNAW, Utrecht, The Netherlands, and the CMW collection of the Forestry and Agricultural Biotechnology Institute, Pretoria,

South Africa). Their nucleotide sequences were downloaded from the GenBank sequence database maintained by the National Center for Biotechnology Information (NCBI) and were included for alignment and phylogenetic analyses. GenBank accession numbers for all sequences are listed in Table 3. Sequences were aligned using ClustalW implemented in MEGA 7 (Kumar et al. 2016) with manual adjustments. Phylogenetic trees for the Botryosphaeriaceae and Diaportheaceae were constructed with MEGA 7 using Maximum Likelihood with the optimal nucleotide substitution model determined by the Akaike Information Criterion (Akaike 1974). Nodal support consisted of nonparametric bootstrapping with 1,000 replicates. All positions containing gaps and missing data were eliminated.

Results

Alignment of 56 DNA sequences of the Botryosphaeriaceae resulted in a dataset of 1028 nucleotide positions (604 positions in the ITS partition and 424 in the EF partition). These included 519 conserved sites (ITS = 385, EF = 134), 260 variable sites (ITS = 112, EF = 148) and 249 parsimony-informative sites (ITS = 107; EF = 142). The optimal model of nucleotide substitution inferred using the AIC was the Tamura-Nei model (Tamura and Nei 1993) with a discrete Gamma distribution (TN93 + G). The tree with the highest log likelihood (-1604.8436) is shown in Figure 1. The alignment of 28 Diaportheaceae DNA sequences comprised 1171 nucleotide positions (575 positions in the ITS partition and 596 in the EF partition), of which 481 were conserved (ITS = 356, EF = 125), 398 were variable (ITS = 129, EF = 269) and 292 parsimony-informative (ITS = 90, EF = 202). The AIC-inferred optimal model of nucleotide substitution was a General Time Reversible model

(Nei and Kumar 2000) with a discrete Gamma distribution (GTR + G). The tree with the highest log likelihood (-3100.69) is shown in Figure 2.

Phylogenetic analyses of the combined ITS and EF sequences assigned the isolates collected from the Merced County almond and walnut orchards to eight putative species belonging to four genera in the Botryosphaeriaceae family and three species in one genus in the Diaportheaceae family. Botryosphaeriaceae species included *Diplodia corticola, Di. mutila*, *Di. seriata*, *Dothiorella iberica/D. sarmentorum*, *Do. viticola, Neofusicoccum parvum*, *Neof. mediterraneum*, and *Neoscytalidium dimidiatum* (Figure 1, Botryosphaeriaceae) and Diaportheaceae species included *Diaporthe ampelina*, *Dia. eres* and *Dia. chamaeropis* (Figure 2, Diaportheaceae). All the species clustered in clades that were supported with strong bootstrap values except for *Do. iberica* and *Do. sarmentorum* that did not segregate into distinct and well supported clades and will be further referred to as *Dothiorella* spp. in this manuscript.

Our results also showed that the majority of the 23 wood samples were infected with *Ceratocystis* (10 samples) followed by Botryosphaeriaceae (7 samples, including *Di. seriata* and *Dothiorella* spp.), Diaportheaceae (4 samples, including *Dia. chamaeropis*), *Eutypa* (1 sample) and *Cytospora* (1 sample). The walnut wood samples were only infected with *Diaporthe* species. *Ceratocystis* and Botryosphaeriaceae taxa were isolated from all three almond orchards. *Eutypa* and *Cytospora* were isolated from the Almond#2 orchard.

Total precipitation from November-April was below average for Merced County (~278 mm CIMIS average per year for the 1999-2019 period) for three of the four years of the field trials (Table 3); these months are typically the wet months in California and

coincide with the time when fungal inoculum causing wood diseases is airborne (Úrbez-Torres et al. 2010). In particular, 2013-2014 was an exceptionally dry year (~159 mm total precipitation) with few wet events during the period of data recording (13 of the 30 weekly recording dates). *Diaporthe* species were trapped almost weekly in the walnut orchard and repeatedly from the same trees whereas they were rarely trapped in almond orchards, even in the almond orchard (#3) adjacent to the walnut orchard. *Diplodia seriata* was the most widely dispersed taxon as it was trapped in all four orchards, albeit randomly and at a low frequency (few trapping events per orchard and never trapped from the same tree). *Dothiorella* spp. incidence in almond orchards was greater than that of *Di. seriata* and *Dothiorella* spp. appeared to prevail in older almond orchards (Almond #1 and #2). *Di. corticola*, *Di. mutila*, *Neos. dimidiatum, Do. viticola* and *Diaporthe* spp. were all specific to individual almond orchards. *Do. viticola* appeared well established in Almond #2 based on more frequent and repeated trapping events from the same tree whereas the other taxa were randomly trapped in single events. The walnut orchard hosted five Botryosphaeriaceae species including *Neof. mediterraneum*, *Neof. parvum*, *Neos. dimidiatum, Di. seriata* and *Di. mutila,* as well as all three *Diaporthe* species. We also trapped species of *Cytospora* sp. and *Eutypa lata* in Almond #2 in a single week in the two years surveyed for each orchard.

Discussion

Spore-traps are commonly deployed to monitor airborne spores. They have been used in the fields of plant pathology to predict the risks of endemic or emerging crop diseases and enact control measures in a timely manner, or in plant biosecurity to prevent

the incursion of regulated diseases (Jackson and Bayliss 2011). Passive spore trapping methods such as the one used in this study (glass slides coated with petroleum jelly) have been largely used in perennial cropping systems to improve wood diseases management guidelines including timing of pruning and fungicide application (Eskalen et al. 2013; Molnar et al. 2020; Trouillas et al. 2012; Urbez-Torres et al. 2010), because they are affordable and allow for the collection of large-scale environmental data (Aguayo et al. 2018). Other studies have relied on active spore trapping devices (i.e., volumetric spore sampler) to study the epidemiology of wood diseases (Billones-Baaijens et al. 2018; Luo et al. 2020; van Niekerk et al. 2010), as active spore trapping devices are more efficient at capturing bioaerosols and integrating accurate weather data in order to build dependable forecasting models (Aguayo et al. 2018; Jackson and Bayliss 2011). Those studies have all established that wet events (rain, irrigation) and high relative humidity (for some taxa) trigger the release of pathogen inoculum in the dormant season, at a time when trees/vines are commonly pruned, which makes them vulnerable to fungal infection (Ahimera et al. 2004; Eskalen et al. 2013; Luo et al. 2020; Michailides and Morgan 1993; Pusey 1989; Trouillas et al. 2012; Urbez-Torres et al. 2010; van Niekerk et al. 2010).

Our data provided evidence that Diaportheaceae and Botryosphaeriaceae spores were mainly airborne after wet events although Diaportheaceae and *Di. seriata* spores were also traps when rain was not recorded by the CIMIS weather station (Supplemental Table). The data also indicated that total precipitations and number of wet events affected spore trapping frequency of fungal taxa (Table 3). For example, *Dothiorella* spp. in Almond #1 and #2, and *Do. viticola* in Almond #2 were more frequently trapped in wet years, while

almost no trapping of fungal spores occurred in the youngest orchard (Almond #3) in a dry year (2014-2015). However, our focus was not to quantitatively record airborne spore inoculum and re-investigate the cause and effect between precipitation events/amounts with pathogen spore counts in orchards, but rather to focus on a qualitative assessment of the fungal inoculum using DNA sequences and test the hypothesis that the characteristics of the orchard impacted airborne pathogen profiles. To that end, we selected four orchards based on crop type, orchard age, neighboring riparian trees and adjacent perennial crops. Our data indicated that orchards were under the pressure of a broad spectrum of fungal pathogens. Fungi belonging to nine genera representing fifteen species were trapped in total, with the Botryosphaeriaceae and Diaportheaceae families being the most frequently represented taxonomic groups. One can argue that this is an underrepresentation of the actual species diversity of the fungal pathogenic airborne population in those orchards, given the methodology used to address our hypothesis. Specifically, culturing coupled with Sanger sequencing for disease diagnosis is time-consuming and can become costly especially with a high number of isolates to sequence. In fact, due to the high number of *Diaporthe* isolates recovered from walnut spore traps we only identified by DNA fingerprint a sub-sample of the trapped *Diaporthe* population and identified three species (three airborne and one from wood samples). This is likely an underrepresentation of the number of *Diaporthe* species occurring in California almond and walnut orchards as many additional species have been identified locally and globally (Chen et al. 2014b; Diogo et al. 2010; Fan et al. 2018; Holland et al. 2020; Jimenez Luna et al. 2020; Lawrence et al. 2015; León et al. 2020; López-Moral et al. 2020b). Another limiting factor is that the

culturing/Sanger sequencing method skews the outcome of the culturable airborne mycobiome towards fast-growing fungi and/or fungi prevalent in a given environment. For example, species of *Pallidophorina* or *Collophorina* were previously found in almond orchards in California but those are not likely to be recovered in culture given their slow-growing nature and lower prevalence in orchards (Holland et al. 2020). Molecular methods (e.g., multiplex qPCR, qPCR using multispecies primers, nested PCR) have recently been coupled with spore trapping devices to improve the detection sensitivity and data accuracy (Billones-Baaijens et al. 2018; Luo et al. 2020; Molnar et al. 2020), but those methods selectively detect taxa that are targeted with the designed primers and overlook the others. Future studies that integrate high-throughput sequencing technologies from environmental samples and use fungal metabarcoding will allow for quick, sensitive and reliable profiling of all the fungal species that are airborne in orchards and vineyards (Aguayo et al. 2018; Castaño et al. 2017).

Our results also highlighted the disparity between spore trapping and wood samples diagnoses. One limit of our approach is that it only captures airborne fungi but overlooks pathogens that use different infection routes. In line with previous reports (Holland et al. 2020) our diagnosis showed that the majority of the wood samples collected from almond orchards were infected with *Ceratocystis*, which is reported to be predominantly vectored by insect (Moller and DeVay 1968). Wood pathogens can also be vectored by mechanical harvesters (Holland et al. 2020), and nursery plant material (Chen et al. 2013a; Marek et al. 2013). Thus, our data warns about using only spore trapping data to predict the risks of canker diseases in orchards or vineyards.

Despite the limitations of our method, the broad diversity of the trapped airborne pathogens coincides with previous reports (Luo et al. 2020; Urbez-Torres et al. 2010; van Niekerk et al. 2010). Our data indicated that airborne pathogenic inoculum profile in orchards was made up of endemic taxa from within the orchard as well as immigrant taxa originating from nearby alternate plant hosts in riparian areas and vineyards/orchards. Immigrant taxa were characterized by rare and random trapping events and we anticipate that those came from cultivated and wild tree hosts at the perimeter of the orchard because Botryosphaeriaceae and Diaportheaceae spores have been reported to travel only short distances (Ahimera et al. 2004; Linders et al. 1996; Michailides and Morgan 1993; Urbez-Torres et al. 2010). In contrast, endemic taxa were characterized by frequent and repeated trapping events from orchards and trees, respectively, and included in our dataset *Dothiorella* spp. (Almond #1 and #2), *Do. viticola* (Almond #2), *Neof. mediterraneum* and *Diaporthe* spp. (Walnut). Some endemic taxa (*Dothiorella* and *Diaporthe* spp.) were also recovered from symptomatic wood samples. *Do. viticola* and *Neof. mediterraneum* were not reisolated perhaps because too few wood samples were collected from orchards.

Walnut appeared to be a favorite tree host to the Diaportheaceae group as spores were frequently and repeatedly trapped in both years of the trial, yet they were rarely trapped in the neighboring or older almond orchards. Several species of *Diaporthe* have been identified from symptomatic trees in walnut orchards worldwide (Chen et al. 2014b; Jimenez Luna et al. 2020; López-Moral et al. 2020a). However, these authors found that overall members of the Botryosphaeriaceae were the most frequently isolated species from walnut trees showing dieback and cankers in both California and Spain. In contrast,

Dothiorella spp. appeared to be more specific to almond orchards and were especially frequently trapped and reisolated from older orchards. *Do. sarmentorum* and *Do. viticola* have been found in almond in California and Iran (Holland et al. 2020; Inderbitzin et al. 2010; Sohrabi et al. 2020). *Dothiorella iberica* has also recently been reported in California (Doll et al. 2015; Holland et al. 2020), but to our knowledge not in other countries. This fungus is a pathogen of many perennial crops in California including grapevine, olive, walnut, pistachio and avocado trees (Chen et al. 2014b; Chen et al. 2014a; Eskalen et al. 2013; Urbez-Torres and Gubler 2009; Urbez-Torres et al. 2013) and likely originated initially from the surrounding vineyards/orchards. Unlike *Diaporthe* and *Dothiorella* spp., *Di. seriata* was not host specific and was trapped and isolated in both walnut and almond orchards, albeit at a low frequency. These results are not surprising given the known broad host range of this fungus, and that it has been commonly isolated from trees in all major almond and walnut producing countries (Chen et al. 2014b; Holland et al. 2020; López-Moral et al. 2020a; Olmo et al. 2016; Sohrabi et al. 2020). Interestingly, all three taxa (*Di. seriata*, *Dothiorella* spp. and *Diaporthe* spp.) have been described as weak pathogens to walnut and almond (Chen et al. 2014b; Holland et al. 2020; López-Moral et al. 2020a) likely because they lack the enzymatic capabilities to colonize woody tissues and break down lignin (Morales-Cruz et al. 2015). Taken together, these results suggest early infection events by these pathogens of the host green tissues (fruit, shoot, leaves) and establishment in orchards after formation of reproductive structures on mummies, leaf litter or twigs and spread by spores to neighboring trees. The epidemiology of these pathogens is in contrast with the more virulent pathogens (e.g., *Neofusicoccum*

species) that can infect lignified tissues of trees (branch, limb, trunk) but need longer time period to colonize their hosts and form fruiting structures on dead wood.

Our data perhaps suggests that orchard age impacts spore trapping profile. It has been well established that incidence of wood canker diseases increases in aging vineyards/orchards (Duthie et al. 1991) and the parallel can be made with spore trapping data. No or little disease pressure was reported in the youngest orchard whereas in older almond orchards, we measured both an increased incidence of endemic fungi as already discussed for *Dothiorella* species and a broader spectrum of pathogenic species. In addition, total precipitation and number of wet events in the dormant season influenced the range and frequency of pathogenic species trapped in orchards. For example, the endemic taxa *Dothiorella* spp. were more frequently trapped in wet vs. dry years in the same orchards (Almond #1 and #2) and a broader array of immigrant taxa were trapped in the young orchard (Almond #3) in a wet vs. dry year. These results are consistent with previous reports indicating a higher disease incidence following intense wet periods and warns about the importance of extreme environmental conditions and the change in weather patterns (e.g., El Niño) in disease outbreaks (Moral et al. 2019b).

Our study highlights once again the importance of the timing of pruning trees for management of canker diseases and support evidence that under dry conditions, infection by airborne fungal vascular pathogens is minimal. Adopting pruning wound management strategies, especially in young orchards, is instrumental to avoid early infection events and decrease chances of endemic infections thereby increasing long-term productivity and profitability as shown for vineyards (Gispert et al. 2020). Additional trapping at a broader

scale that integrates all possible infection routes, coupled with non-selective, sensitive, and rapid molecular diagnostic methods will help refine the disease risk prediction to enact adapted management strategies.

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References

Abdollahzadeh, J., Javadi, A., Zare, R., Phillips, A. J. L. 2014. A phylogenetic study of Dothiorella and Spencermartinsia species associated with woody plants in Iran, New Zealand, Portugal and Spain. *Persoonia* 32:1-12.

Adaskaveg, J. E., Forster, H., Connell, J. H. 1999. First Report of Fruit Rot and Associated Branch Dieback of Almond in California Caused by a Phomopsis Species Tentatively Identified as P. amygdali. *Plant Disease* 83:1073.

Aguayo, J., Fourrier-Jeandel, C., Husson, C., Ioos, R. 2018. Assessment of Passive Traps Combined with High-Throughput Sequencing To Study Airborne Fungal Communities. *Applied Environmental Microbiology* 84.

Ahimera, N., Gisler, S., Morgan, D. P., Michailides, T. J. 2004. Effects of single-drop impactions and natural and simulated rains on the dispersal of Botryosphaeria dothidea conidia. *Phytopathology* 94:1189-1197.

Akaike, H. 1974. A new look at the statistical model identification. IEEE Transactions on Automatic Control 19:716-723.

Billones-Baaijens, R., Urbez-Torres, J. R., Liu, M. F., Ayres, M., Sosnowski, M., Savocchia, S. 2018. Molecular Methods to Detect and Quantify Botryosphaeriaceae Inocula Associated With Grapevine Dieback in Australia. *Plant Disease* 102:1489-1499.

Castano, C., Oliva, J., de Aragon, J. M., Alday, J. G., Parlade, J., Pera, J., Bonet, J. A. 2017. Mushroom Emergence Detected by Combining Spore Trapping with Molecular Techniques. *Applied Environmental Microbiology* 83.

CDFA. 2019. California agricultural production statistics. California Department of Food and Agriculture. https://www.cdfa.ca.gov/statistics/.

Chen, S. F., Morgan, D. P., Michailides, T. J. 2014a. Botryosphaeriaceae and Diaporthaceae associated with panicle and shoot blight of pistachio in California, USA. *Fungal Diversity* 67:157-179.

Chen, S. F., Fichtner, E., Morgan, D. P., Michailides, T. J. 2013a. First Report of Lasiodiplodia citricola and Neoscytalidium dimidiatum Causing Death of Graft Union of English Walnut in California. *Plant Disease* 97:993-993.

Chen, S. F., Morgan, D., Beede, R. H., Michailides, T. J. 2013b. First Report of Lasiodiplodia theobromae Associated with Stem Canker of Almond in California. *Plant Disease* 97:994-995.

Chen, S. F., Morgan, D. P., Hasey, J. K., Anderson, K., Michailides, T. J. 2014b. Phylogeny, Morphology, Distribution, and Pathogenicity of Botryosphaeriaceae and Diaporthaceae from English Walnut in California. *Plant Disease* 98:636-652.

Diogo, E. L. F., Santos, J. M., Phillips, A. J. L. 2010. Phylogeny, morphology and pathogenicity of Diaporthe and Phomopsis species on almond in Portugal. *Fungal Diversity* 44:107-115.

Doll, D., Michailides, T., Rolshausen, P. 2013. Botryosphaeriaceae associated with almond trunk cankers: A threat to the almond industry? *Phytopathology* 103:13-13.

Doll, D. A., Rolshausen, P. E., Pouzoulet, J., Michailides, T. J. 2015. First Report of Dothiorella iberica Causing Trunk and Scaffold Cankers of Almond in California. *Plant Disease* 99:1185-1185.

Duthie, J. A., Munkvold, G. P., Marois, J. J. 1991. Relationship between age of vineyard and incidence of Eutypa dieback. *Phytopathology* 81:1183.

English, H., Davis, J. R., Devay, J. E. 1966. Dothiorella Canker a New Disease of Almond Trees in California. *Phytopathology* 56:146.

Eskalen, A., Faber, B., Bianchi, M. 2013. Spore Trapping and Pathogenicity of Fungi in the Botryosphaeriaceae and Diaporthaceae Associated with Avocado Branch Canker in California. *Plant Disease* 97:329-332.

Fan, X. L., Yang, Q., Bezerra, J. D. P., Alvarez, L. V., Tian, C. M. 2018. Diaporthe from walnut tree (*Juglans regia*) in China, with insight of the Diaporthe eres complex. Mycological Progress 17:841-853.

Gispert, C., Kaplan, J. D., Deyett, E., Rolshausen, P. E. 2020. Long-Term Benefits of Protecting Table Grape Vineyards against Trunk Diseases in the California Desert. Agronomy-Basel 10.

Gramaje, D., Agusti-Brisach, C., Perez-Sierra, A., Moralejo, E., Olmo, D., Mostert, L., Damm, U., Armengol, J. 2012. Fungal trunk pathogens associated with wood decay of almond trees on Mallorca (Spain). *Persoonia* 28:1-13.

Henderson, B., Sosnowski, M. R., McCarthy, M. G., Scott, E. S. 2021. Incidence and severity of Eutypa dieback in grapevines are related to total surface area of pruning wounds. Aust J Grape Wine R 27:87-93.

Holland, L. A., Lawrence, D. P., Nouri, M. T., Travadon, R., Harrington, T. C., Trouillas, F. P. 2019. Taxonomic revision and multi-locus phylogeny of the North American clade of Ceratocystis. *Fungal Systematics Evolution* 3:135-156.

Holland, L. A., Nonni, M. T., Crespo, M. P., Holtz, B. A., Yaghmour, M. A., Doll, D. A., Trouillas, F. P. 2018. First Report of Collophora hispanica and C. paarla Causing Branch Cankers of Almond in California. *Plant Disease* 102:1663-1664.

Holland, L. A., Trouillas, F. P., Nouri, M. T., Lawrence, D. P., Crespo, M., Doll, D. A., Duncan, R., Holtz, B. A., Culumber, C. M., Yaghmour, M., Niederholzer, F., Lightle, D., Jarvis-Shean, K. S., Gordon, P. E., Fichtner, E. J. 2020. Fungal Pathogens Associated with Canker Diseases of Almond in California. *Plant Disease*.

Inderbitzin, P., Bostock, R. M., Trouillas, F. P., Michailides, T. J. 2010. A six locus phylogeny reveals high species diversity in Botryosphaeriaceae from California almond. *Mycologia* 102:1350-1368.

Jackson, S. L., Bayliss, K. L. 2011. Spore traps need improvement to fulfil plant biosecurity requirements. *Plant Pathology* 60:801-810.

Jacobs, K., Bergdahl, D. R., Wingfield, M. J., Halik, S., Seifert, K. A., Bright, D. E., Wingfield, B. D. 2004. Leptographium wingfieldii introduced into North America and found associated with exotic Tomicus piniperda and native bark beetles. *Mycologia Restoration* 108:411-418.

Jimenez Luna, I., Cadiz, F., Aravena, R., Larach, A., Besoain, X., Ezcurra, E., Rolshausen, P. E. 2020. First Report of Diaporthe cynaroidis and D. australafricana Associated with Walnut Branch Canker in Chile. *Plant Disease* 104:2732-2733.

Kolarik, M., Freeland, E., Utley, C., Tisserat, N. 2011. Geosmithia morbida sp nov., a new phytopathogenic species living in symbiosis with the walnut twig beetle (Pityophthorus juglandis) on Juglans in USA. *Mycologia* 103:325-332.

Kumar, S., Stecher, G., and Tamura, K. 2016. MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. *Molecular Biology Evolution* 33:1870-1874.

Lawrence, D. P., Travadon, R., Baumgartner, K. 2015. Diversity of Diaporthe species associated with wood cankers of fruit and nut crops in northern California. *Mycologia* 107:926-940.

Lawrence, D. P., Holland, L. A., Nouri, M. T., Travadon, R., Abramians, A., Michailides, T. J., Trouillas, F. P. 2018. Molecular phylogeny of Cytospora species associated with canker diseases of fruit and nut crops in California, with the descriptions of ten new species and one new combination. *Ima Fungus* 9:333-370.

Leon, M., Berbegal, M., Rodriguez-Reina, J. M., Elena, G., Abad-Campos, P., Ramon-Albalat, A., Olmo, D., Vicent, A., Luque, J., Miarnau, X., Agusti-Brisach, C., Trapero, A., Capote, N., Arroyo, F. T., Aviles, M., Gramaje, D., Andres-Sodupe, M., Armengol, J. 2020. Identification and Characterization of Diaporthe spp. Associated with Twig Cankers and Shoot Blight of Almonds in Spain. Agronomy-Basel 10.

Leslie, C., Seybold, S. J. Graves, A., Cranshaw, W., Tisserat, N. 2009. Potential impacts of thousand cankers disease on commercial walnut production and walnut germplasm conservation. *Acta horticulturae* 861: 431-434. 10.17660/ActaHortic.2010.861.59.

Linders, E. G. A., VanDamme, J. M. M., Zadoks, J. C. 1996. Transmission and overseasoning of Diaporthe adunca on Plantago lanceolata. *Plant Pathology* 45:59-69.

Lopez-Moral, A., Lovera, M., Raya, M. D., Cortes-Cosano, N., Arquero, O., Trapero, A., Agusti-Brisach, C. 2020a. Etiology of Branch Dieback and Shoot Blight of English Walnut Caused by Botryosphaeriaceae and Diaporthe Species in Southern Spain. *Plant Disease* 104:533-550.

Lopez-Moral, A., Raya, M. D., Ruiz-Blancas, C., Medialdea, I., Lovera, M., Arquero, O., Trapero, A., Agusti-Brisach, C. 2020b. Aetiology of branch dieback, panicle and shoot blight of pistachio associated with fungal trunk pathogens in southern Spain. *Plant Pathology* 69:1237-1269.

Luo, Y., Niederholzer, F. J. A., Felts, D. G., Puckett, R. D., Michailides, T. J. 2020. Inoculum quantification of canker-causing pathogens in prune and walnut orchards using real-time PCR. *Journal of Applied Microbiology* 129:1337-1348.

Luo, Y., Lichtemberg, P., Niederholzer, F., Lightle, D. M., Felts, D. G., and Michailides, T. J. 2019. Understanding the Process of Latent Infection of Canker-Causing Pathogens in Stone Fruit and Nut Crops in California. *Plant disease* 103(9): 2374–2384. https://doi.org/10.1094/PDIS-11-18-1963-RE

Marek, S. M., Yaghmour, M. A., Bostock, R. M. 2013. Fusarium spp., Cylindrocarpon spp., and Environmental Stress in the Etiology of a Canker Disease of Cold-Stored Fruit and Nut Tree Seedlings in California. *Plant Disease* 97:259-270.

Michailides, T. J., Morgan, D. P. 1993. Spore Release by Botryosphaeria-Dothidea in Pistachio Orchards and Disease-Control by Altering the Trajectory Angle of Sprinklers. *Phytopathology* 83:145-152.

Moller, W. J., DeVay, J. E. 1968. Insect transmission of Ceratocystis fimbriata in decidious fruit orchards. *Phytopathology* 58:1499-1508.

Molnar, M., Voegele, R. T., Fischer, M. 2020. Grapevine trunk diseases in German viticulture IV. Spreading of spores of Phaeomoniella chlamydospora in Esca-affected vineyards. *Vitis* 59:63-69.

Moral, J., Morgan, D., Michailides, T. J. 2019a. Management of Botryosphaeria canker and blight diseases of temperate zone nut crops. *Crop Protection* 126.

Moral, J., Morgan, D., Trapero, A., Michailides, T. J. 2019b. Ecology and Epidemiology of Diseases of Nut Crops and Olives Caused by Botryosphaeriaceae Fungi in California and Spain. *Plant Disease* 103:1809-1827.

Morales-Cruz, A., Amrine, K. C. H., Blanco-Ulate, B., Lawrence, D. P., Travadon, R., Rolshausen, P. E., Baumgartner, K., Cantu, D. 2015. Distinctive expansion of gene families associated with plant cell wall degradation, secondary metabolism, and nutrient uptake in the genomes of grapevine trunk pathogens. *Bmc Genomics* 16.

Munkvold, G. P., Duthie, J. A., Marois, J. J. 1994. Reductions in Yield and Vegetative Growth of Grapevines Due to Eutypa Dieback. *Phytopathology* 84:186-192.

Nei, M., Kumar, S. 2000. Molecular Evolution and Phylogenetics. Oxford University Press, New York.

Nouri, M. T., Lawrence, D. P., Yaghmour, M. A., Michailides, T. J., Trouillas, F. P. 2018. Neoscytalidium dimidiatum Causing Canker, Shoot Blight and Fruit Rot of Almond in California. *Plant Disease* 102:1638-1647.

Olmo, D., Armengol, J., Leon, M., Gramaje, D. 2016. Characterization and Pathogenicity of Botryosphaeriaceae Species Isolated from Almond Trees on the Island of Mallorca (Spain). *Plant Disease* 100:2483-2491.

Pearce, R. B. 1996. Antimicrobial defences in the wood of living trees. *New Phytologist* 132:203-233.

Pouzoulet, J., Pivovaroff, A. L., Santiago, L. S., Rolshausen, P. E. 2014. Can vessel dimension explain tolerance toward fungal vascular wilt diseases in woody plants? Lessons from Dutch elm disease and esca disease in grapevine. *Frontiers in Plant Science* 5.

Pusey, P. L. 1989. Availability and Dispersal of Ascospores and Conidia of Botryosphaeria in Peach Orchards. *Phytopathology* 79:635-639.

Shigo, A. L., Marx, H. G. 1977. Compartmentalization of Decay in Trees. U S Department of Agriculture Agriculture Information Bulletin 405:1-73.

Sohrabi, M., Mohammadi, H., Leon, M., Armengol, J., Banihashemi, Z. 2020. Fungal pathogens associated with branch and trunk cankers of nut crops in Iran. *European Journal Plant Pathology* 157:327-351.

Tamura, K., and Nei, M. 1993. Estimation of the Number of Nucleotide Substitutions in the Control Region of Mitochondrial-DNA in Humans and Chimpanzees. *Molecular Biology Evolution* 10:512-526.

Teviotdale, Beth & Harper, Dennis. 1996. Almond pruning wounds, bark abrasions susceptible to Ceratocystis. *California Agriculture* 50: 29-33. 10.3733/ca.v050n03p29.

Trouillas, F. P., Gubler, W. D. 2010. Host Range, Biological Variation, and Phylogenetic Diversity of Eutypa lata in California. *Phytopathology* 100:1048-1056.

Trouillas, F. P., Peduto, F., Lorber, J. D., Sosnowski, M. R., Grant, J., Coates, W. W., Anderson, K. K., Caprile, J., Gubler, W. D. 2012. Calosphaeria Canker of Sweet Cherry Caused by Calosphaeria pulchella in California and South Australia. *Plant Disease* 96:648-658.

Urbez-Torres, J. R., Gubler, W. D. 2009. Pathogenicity of Botryosphaeriaceae Species Isolated from Grapevine Cankers in California. *Plant Disease* 93:584-592.

Urbez-Torres, J. R., Peduto, F., Vossen, P. M., Krueger, W. H., Gubler, W. D. 2013. Olive Twig and Branch Dieback: Etiology, Incidence, and Distribution in California. *Plant Disease* 97:231-244.

Urbez-Torres, J. R., Battany, M., Bettiga, L. J., Gispert, C., McGourty, G., Roncoroni, J., Smith, R. J., Verdegaal, P., Gubler, W. D. 2010. Botryosphaeriaceae Species Spore-Trapping Studies in California Vineyards. *Plant Disease* 94:717-724.

USDA-NASS. 2019. Statistics of fruits, tree nuts, and horticultural specialties. United States Department of Agriculture-National Agricultural Statistics Service https://www.nass.usda.gov/Publications/Ag_Statistics/.

Van Niekerk, J. M., Calitz, F. J., Halleen, F., and Fourie, P. H. 2010. Temporal spore dispersal patterns of grapevine trunk pathogens in South Africa. *European Journal Plant Pathology* 127:375-390.

White, T. J., Bruns, T., Lee, S., Taylor, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics.315-322.

Wunderlich, N., Costa, S. S., Tpoi, R. P., Ash, G. J. 2012. First report of Botryosphaeria dothidea causing shoot blight and cankers of pistachio in Australia. *Australasian Plant Disease* 7:47-49.

Tables

Table 2.1 Botryosphaeriaceae and Diaportheaceae fungal collection. Taxa were obtained from the GenBank database and used in phylogenetic analyses to assigned species name for isolates cultured from California almond and walnut orchards.

Identity	Collection code	Host	Country of Origin	GenBank Accession	
				ITS	EF
Diaporthe ambigua	CBS 114015	*Pyrus communis*	South Africa	AF230767	GQ250299
Diaporthe ampelina	CBS 111888	*Vitis vinifera*	USA	KC343016	KC343742
Diaporthe ampelina	CBS 114016	*Vitis vinifera*	France	AF230751	GQ250351
Diaporthe ampelina	UCR-Pho18	*Juglans regia*	CA, USA	MW363167	MW464417
Diaporthe amygdali	CBS 126679	*Prunus dulcis*	Portugal	GQ281791	JX275400
Diaporthe angelicae	CBS 111592	*Heracleum sphondylium*	Austria	FJ889448	GQ250324
Diaporthe aspalathi	CBS 117169	*Aspalathus linearis*	South Africa	DQ286275	DQ286249
Diaporthe chamaeropis	CBS 454.81	*Chamaerops humilis*	Greece	KC343048	KC343048
Diaporthe chamaeropis	CBS 753.70	*Spartium junceum*	Croatia	KC343049	KC343775
Diaporthe chamaeropis	UCR-K1583	*Prunus dulcis*	CA, USA	MW363168	MW464418
Diaporthe cotoneastri	CBS 439.82	*Cotoneaster* sp.	Scotland	FJ889450	GQ250341
Diaporthe crotalariae	CBS 162.33	*Crotalaria spectabilis*	-	FJ889445	GQ250307
Diaporthe cuppatea	CBS 117499	*Aspalathus linearis*	South Africa	GQ250190	GQ250311
Diaporthe eres	CBS 101742	*Fraxinus* sp.	Netherlands	KC343073	KC343799
Diaporthe eres	UCR-Pho19	*Juglans regia*	CA, USA	MW363169	MW464419
Diaporthe eres	UCR-Pho20	*Juglans regia*	CA, USA	MW363170	MW464420

Diaporthe foeniculina	CBS 187.27	*Camellia sinensis*	Italy	MH854926	KC343833
Diaporthe foeniculina	CBS 171.78	*Prunus amygdalus*	Italy	KC343106	KC343832
Diaporthe helianthi	CBS 592.81	*Helianthus annuus*	Serbia	AY705842	GQ250308
Diaporthe longispora	CBS 194.36	*Ribes* sp.	-	KC343135	KC343861
Diaporthe lusitanicae	CBS 123212	*Foeniculum vulgarae*	Portugal	AF439626	GQ250350
Diaporthe melonis	CBS 507.78	*Cucumis melo*	USA	FJ889447	GQ250314
Diaporthe neotheicola	CBS 123208	*Foeniculum vulgare*	Portugal	EU814480	GQ250315
Diaporthe phoenicicola	CBS 161.64	*Areca catechu*	India	FJ889452	GQ250349
Diaporthe rudis	CBS 113201	*Vitis vinifera*	Portugal	KC343234	KC343960
Diaporthe rushicola	6I14	*Prunus dulcis*	CA, USA	KF778872	KF779062
Diaporthe sclerotioides	CBS 296.67	*Cucumis sativus*	Netherlands	FJ889449	GQ250325
Diaporthe stewartii	CBS 193.36	*Cosmos bipinnatus*	-	AY339322	AY339354
Diplodia corticola	CBS 112545	*Quercus suber*	Spain	AY259089	AY573226
Diplodia corticola	CBS 112549	*Quercus suber*	Portugal	AY259100	AY573227
Diplodia corticola	UCR-DC1	*Prunus dulcis*	CA, USA	MW393554	MW496392
Diplodia mutila	4D33	*Persea americana*	CA, USA	KF778789	KF778979
Diplodia mutila	CBS 112553	*Vitis vinifera*	Portugal	AY259093	AY573219
Diplodia mutila	CBS 230.30	*Phoenix dactylifera*	USA	DQ458886	DQ458869
Diplodia mutila	CMW7060	*Fraxinus excelsior*	Netherlands	AY236955	AY236904
Diplodia mutila	UCR-BS2	*Prunus dulcis*	CA, USA	MW393549	MW496387
Diplodia mutila	UCR-BS3	*Prunus dulcis*	CA, USA	MW393550	MW496388

Diplodia mutila	UCR-DM1	*Juglans regia*	CA, USA	MW393559	MW496398
Diplodia mutila	UCR-DM2	*Juglans regia*	CA, USA	MW393561	MW496399
Diplodia mutila	UCR-DM3	*Prunus dulcis*	CA, USA	MW393562	MW496400
Diplodia seriata	2K33	*Punica granatum*	CA, USA	KF778795	KF778985
Diplodia seriata	3K67	*Juglans regia*	CA, USA	KF778797	KF778987
Diplodia seriata	UCR-DS12	*Prunus dulcis*	CA, USA	MW393565	MW496403
Diplodia seriata	UCR-DS13	*Prunus dulcis*	CA, USA	MW393566	MW496404
Diplodia seriata	UCR-DS14	*Juglans regia*	CA, USA	MW393567	MW496405
Diplodia seriata	UCR-DS15	*Prunus dulcis*	CA, USA	MW393568	MW496406
Diplodia seriata	UCR-K1581	*Prunus dulcis*	CA, USA	MW393569	MW496407
Diplodia seriata	UCR-K1582	*Prunus dulcis*	CA, USA	MW393570	MW496408
Diplodia seriata	UCR-K1586	*Prunus dulcis*	CA, USA	MW393571	MW496409
Diplodia seriata	UCR-K1590	*Prunus dulcis*	CA, USA	MW393572	MW496410
Dothiorella iberica	5G97	*Juglans regia*	CA, USA	KF778808	KF778998
Dothiorella iberica	CBS 115041	Quercus ilex	Spain	GU251168	GU251300
Dothiorella sarmentorum	CBS 115038	*Malus pumila*	Netherlands	AY573206	AY573223
Dothiorella sarmentorum	PD280	*Ulmus* sp.	Great Britain	GU251171	GU251303
Dothiorella sarmentorum	PD78	*Prunus dulcis*	CA, USA	GU251169	GU251301
Dothiorella spp.	UCR-DoS2	*Prunus dulcis*	CA, USA	MW393563	MW496401
Dothiorella spp.	UCR-DoS3	*Prunus dulcis*	CA, USA	MW393564	MW496402
Dothiorella spp.	UCR-Dsp1	*Prunus dulcis*	CA, USA	MW393573	MW496411

Dothiorella spp.	UCR-Dsp2	*Prunus dulcis*	CA, USA	MW393574	MW496412
Dothiorella spp.	UCR-DI1	*Prunus dulcis*	CA, USA	MW393555	MW496393
Dothiorella spp.	UCR-DI3	*Prunus dulcis*	CA, USA	MW393556	MW496394
Dothiorella spp.	UCR-DI5	*Prunus dulcis*	CA, USA	MW393557	MW496395
Dothiorella spp.	UCR-DI6	*Prunus dulcis*	CA, USA	MW393558	MW496396
Dothiorella spp.	UCR-K1589	*Prunus dulcis*	CA, USA	MW393559	MW496397
Neofusicoccum mediterraneum	CBS 121558	*Olea europea*	Italy	GU251175	GU251307
Neofusicoccum mediterraneum	CBS 121718	*Eucalyptus* sp.	Greece	GU251176	GU251308
Neofusicoccum mediterraneum	UCR-NM2	*Juglans regia*	CA, USA	MW393578	MW496416
Neofusicoccum mediterraneum	UCR-NM3	*Juglans regia*	CA, USA	MW393579	MW496417
Neofusicoccum parvum	CBS 110301	*Vitis vinifera*	Portugal	AY259098	AY573221
Neofusicoccum parvum	CMW 9081	*Populus nigra*	New Zealand	AY236943	AY236888
Neofusicoccum parvum	PD286	*Populus nigra*	New Zealand	GU251125	GU251257
Neofusicoccum parvum	UCR-NP4	*Juglans regia*	CA, USA	MW393580	MW496418
Neofusicoccum parvum	UCR-NP5	*Juglans regia*	CA, USA	MW393581	MW496419
Neoscytalidium dimidiatum	CBS 145.78	-	United Kingdom	KF531816	KF531795
Neoscytalidium dimidiatum	KARE1791	*Prunus dulcis*	CA, USA	MG021578	MG021531
Neoscytalidium dimidiatum	PD103	*Ficus carica*	CA, USA	GU251106	GU251238
Neoscytalidium dimidiatum	UCR-Neo8	*Prunus dulcis*	CA, USA	MW393576	MW496414
Neoscytalidium dimidiatum	UCR-NH1	*Juglans regia*	CA, USA	MW393577	MW496415
Spencermartinsia viticola	CBS 117009	*Vitis vinifera*	Spain	GU251166	GU251298

Spencermartinsia viticola	PD74	*Citrus sp.*	CA, USA	GU251167	GU251299
Spencermartinsia viticola	UCR-BV1	*Prunus dulcis*	CA, USA	MW393551	MW496389
Spencermartinsia viticola	UCR-BV2	*Prunus dulcis*	CA, USA	MW393552	MW496390
Spencermartinsia viticola	UCR-BV3	*Prunus dulcis*	CA, USA	MW393553	MW496391
Spencermartinsia viticola	UCR-DV1	*Prunus dulcis*	CA, USA	MW393575	MW496413

Table 2.2 Metadata of the four orchards where the spore trapping experiments were conducted; orchard type, year of the trial, total precipitations and number of wet events from November 1st to April 30th, fungal taxa (based on ITS and EF sequences) trapped, the number of trapping events (weeks and trees) that each taxon was trapped during that sampling period, and the trees from which the same taxon was trapped multiple times (≥ 2 trapping events). *Di. = Diplodia*, *Nf. = Neofusicoccum*, *Ns. = Neoscytalidium*, *S. = Spencermartinsia.*

Orchard Type	Orchard Age (years)	Trial Year	Number of Wet Events[a]	Total Precipitation (mm)[a]	Taxa Name	Number of Trapping Events (weeks)[b]	Number of Trapping Events (trees)[c]	Number of Trees with Repeated Trapping Events[d]
Almond #1	10	2012-13	22	164.8	*Diaporthe* sp.	1	1	
					Dothiorella spp.	11	16	4
					Ns. dimidiatum	1	1	
		2013-14	13	158.9	*Di. corticola*	1	1	
					Di. seriata	1	1	
					Dothiorella spp.	4	7	2
Almond #2	6	2013-14	13	158.9	*Cytospora* sp.	1	1	
					Di. seriata	1	1	
					Dothiorella spp.	1	1	
					S. viticola	2	2	1
		2014-15	20	214.6	*Dothiorella* spp.	6	6	1
					Eutypa lata	1	1	
					S. viticola	4	4	1
Almond #3	4	2014-15	21	152	Diaporthe sp.	1	1	
		2015-16	24	435.4	*Diaporthe* sp.	2	2	
					Di. mutila	1	1	
					Di. seriata	1	1	
					Dothiorella spp.	1	1	
Walnut	15	2014-15	21	152	*Diaporthe* spp.	18	45	8
					Di. mutila	2	2	
					Di. seriata	3	3	
					Nf. mediterraneum	3	3	1
		2015-16	24	435.4	*Diaporthe* spp.	17	38	9
					Di. mutila	2	2	1
					Di. seriata	4	4	
					Nf. parvum	1	1	
					Ns. dimidiatum	1	1	

[a]based on 30 weekly reports from November 1st to April 30th each year.
[b]based on the 30 weekly trapping events from November 1st to April 30th each year, from any of the 9 trees sampled.
[c]based on a total of 270 trees (30 trapping events x 9 trees per event)
[d]≥2 trapping events from the same tree (9 trees total)

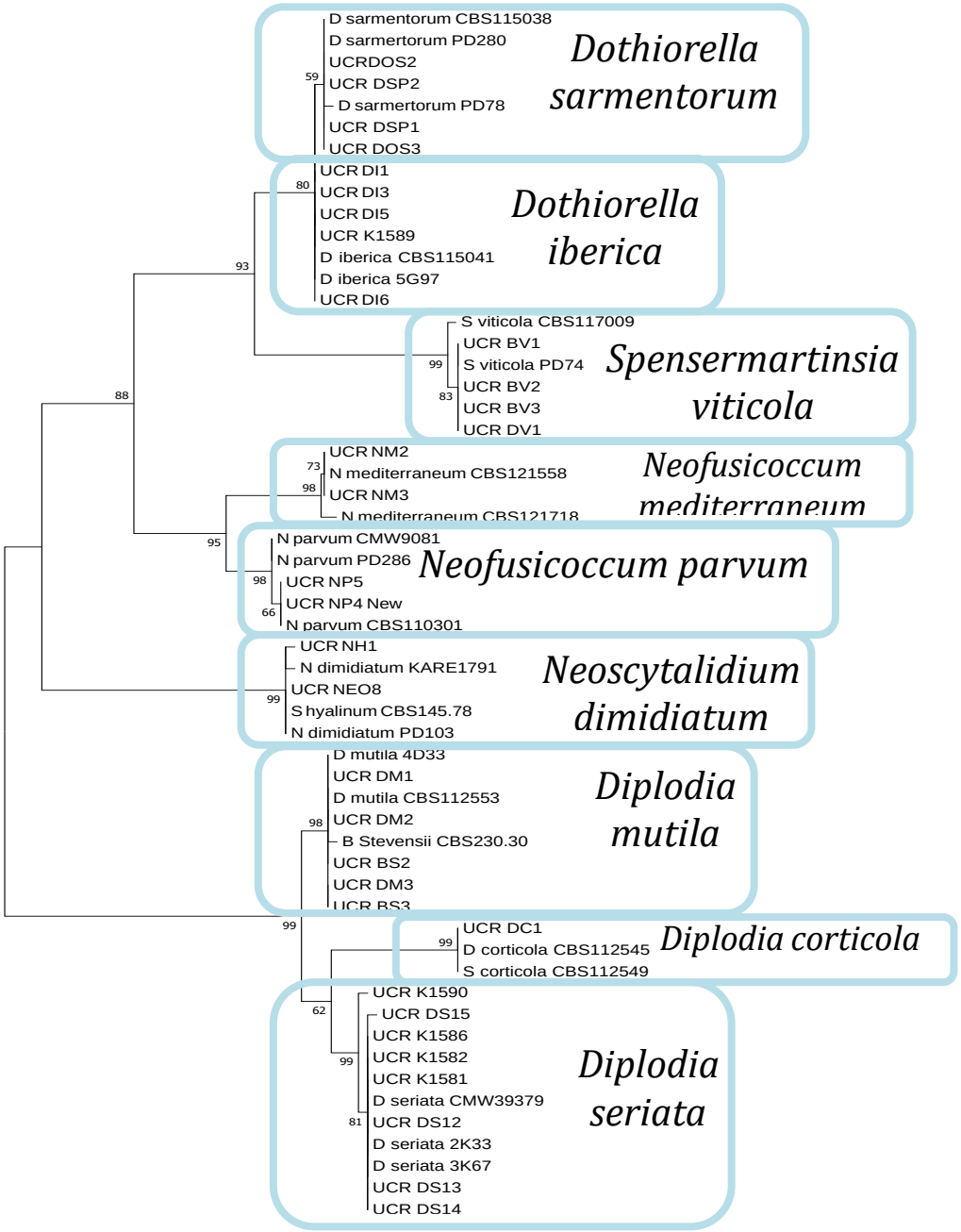


Figure 2.1 Phylogenetic tree reconstructed by maximum likelihood analysis from the concatenated sequences of the nuclear ribosomal internal transcribed spacer and translation elongation factor for 33 Botryosphaeriaceae taxa isolated from three almond orchards and one walnut orchard in Merced County, CA, and 23 reference sequences retrieved from the GenBank database. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Numbers above branches are bootstrap support values (values < 70% not shown).

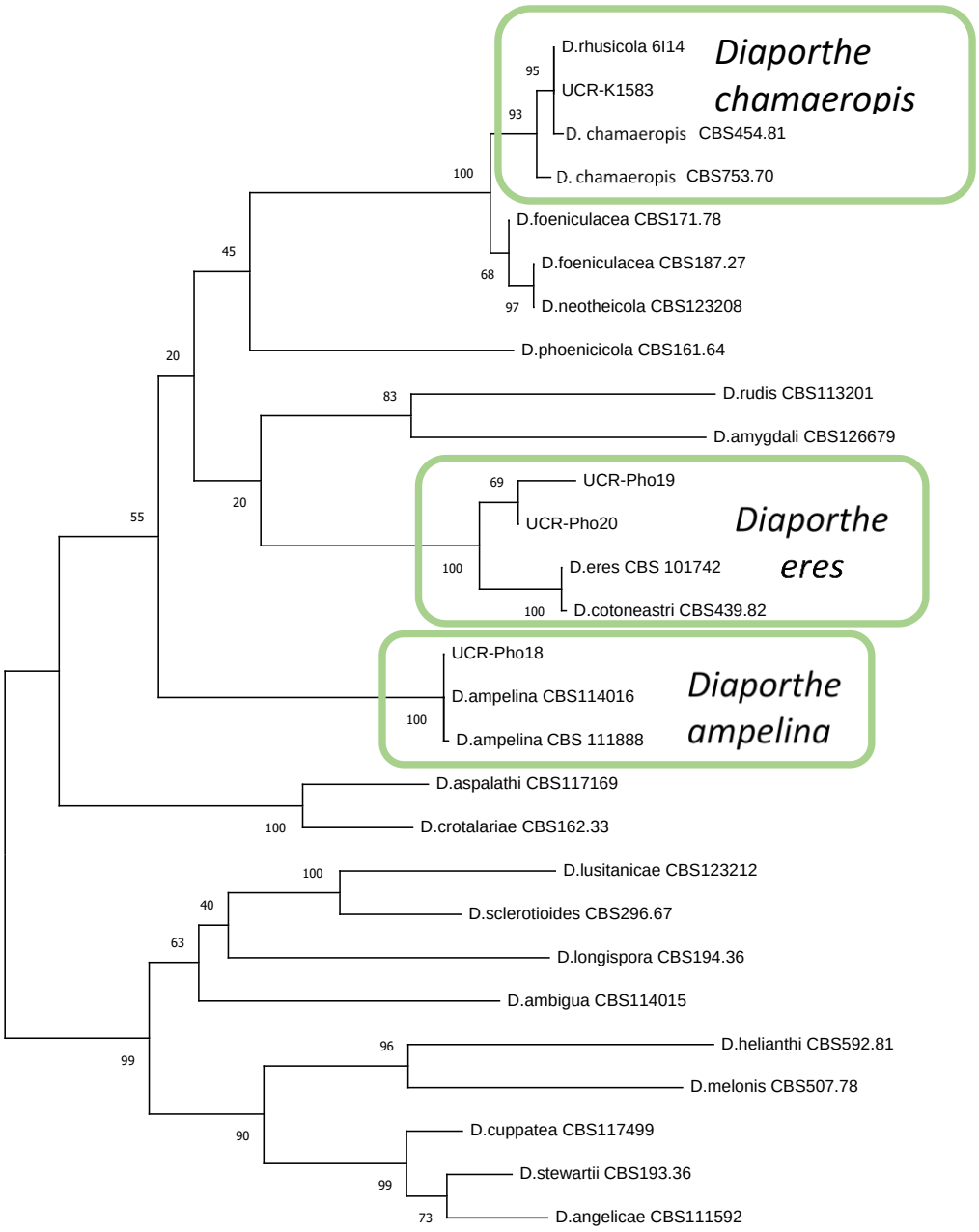


Figure 2.2 Phylogenetic tree reconstructed by maximum likelihood analysis from the concatenated sequences of the nuclear ribosomal internal transcribed spacer and translation elongation factor for four Diaportheaceae taxa recovered from three almond orchards and one walnut orchard in Merced County, CA, and 24 *Diaporthe* reference sequences retrieved from the GenBank database. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Numbers above branches are bootstrap support values (values < 70% not shown).

Supplemental Tables

Table S2.1 Weekly spore trapping of fungal inocula and rain amount (mm; https://cimis.water.ca.gov/) over a 30 weeks period (from October 1st to April 30th) in four orchards for two consecutive years. No data in the week column indicate no rain events and/or no trapped inoculum. Cy: *Cytospora* sp.; Dc: *Diplodia corticola*; Di: *Diaporthe* spp.; Do: *Dothiorella* spp.; Dm: *Diplodia mutila*; Ds: *Diplodia seriata*; El: *Eutypa lata*; Nd: *Neoscytalidium dimidiatum*; Nm: *Neofusicoccum mediterraneum*; Np: *Neofusicoccum parvum*; Sv: *Spencermartinsia viticola*. Colored square indicate taxa were trapped from multiple trees. Striped square indicate taxa were previously trapped from the same tree.

Location	Year	Variables	Week																												
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
Almond #1	1	Rain (mm)	0.5		0.5	1.3	2.5	5.1	7.4	7.1	10.9	3.6	26.9	32.5	12.4	9.1		1	0.3	2		7.1		2.5		1.3	4.6	25.4	0.8			
		Inoculum							Do	Do Di	Do		Do			Do			Do Nd			Do	Do	Do				Do	Do			
	2	Rain (mm)							20.6	0.5	8.1								9.4	13.2	4.8	1	41.4	11.4	1.8		27.9	11.4			7.4	
		Inoculum									Do									Dc		Do Ds	Do					Do				
Almond #2	1	Rain (mm)							20.6	0.5	8.1								9.4	13.2	4.8	1	41.4	11.4	1.8		27.9	11.4			7.4	
		Inoculum				Ds			Sv															Sv	Do							Cy
	2	Rain (mm)			1.8	23.9		6.4	3.6	11.9	30	51.3	28.7	1		0.3	0.8	0.5	1	9.4	6.6	1	15.5		3.6				9.9		7.4	
		Inoculum				Do			Do				Do								Do	Do	Sv		Sv El				Sv		Do Sv	
Almond #3	1	Rain (mm)			0.5	24.9	0.5	6.1	2.5	3	22.6	14.5	12.2	2			0.8	0.3	1	21.1	7.4	0.5	1	1.3	1.3				13		15.5	
		Inoculum													Di																	
	2	Rain (mm)						30.2	4.6	6.9	1.5	5.6	15.5	37.8	0.5	34.3	5.1	77.2	4.1	12.7	0.8	9.9	0.5	45.2	41.7	20.8	1		66.3	1	6.6	5.6
		Inoculum							Ds											Do						Di			Dm			Di
Walnut	1	Rain (mm)			0.5	24.9	0.5	6.1	2.5	3	22.6	14.5	12.2	2			0.8	0.3	1	21.1	7.4	0.5	1	1.3	1.3				13		15.5	
		Inoculum				Nm Di		Di	Nm Di	Di	Di		Di	Di	Di			Di		Di	Nm Ds		Di	Ds Dm Di	Di	Di			Di	Di	Ds Dm Di	Di
	2	Rain (mm)						30.2	4.6	6.9	1.5	5.6	15.5	37.8	0.5	34.3	5.1	77.2	4.1	12.7	0.8	9.9	0.5	45.2	41.7	20.8	1		66.3	1	6.6	5.6
		Inoculum		Di			Di	Di	Di	Di		Nd					Np Di	Ds Di	Dm Di	Ds Di	Di				Ds	Ds Di	Di	Di	Di	Di	Di	Dm Di

Chapter III

The effects of water deficit irrigation and pathogen interaction on physiological and morphological traits, midday water potential and canopy carbon-water relations of young walnut trees (*Juglans regia*)

Abstract

Water scarcity and quality, frequent drought events, and the presence of fungal canker diseases impose a threat to walnut production worldwide. Research studies have reported tree crop responses to either abiotic or biotic stress, and the impact on plant morphology, physiology, and water relations. However, the combined effect of water stress and fungal disease on walnut tree growth is still unknown. We hypothesize that (a) water stress treatments will increase the susceptibility of walnut trees to canker disease, (b) cankers will negatively impact walnut tree growth, physiological traits, and (c) plant water potential and the degree of severity of canker diseases will vary across the different water regimes. Using a whole-tree experimental approach, we evaluated morphological and, physiological characteristics, as well as water relations of 2-year-old walnut tree under three water stress regimes (100%, 75% and 25% soil water content) post-inoculated by *Diplodia mutila* and *Neofusicoccum parvum* fungal pathogens. The results reveal that as water stress increased, walnut tree physiological traits [photosynthesis (A), transpiration (E), Stomatal conductance (G_s) and chlorophyll fluorescence (F_v/F_m)], biomass and water potential are negatively impacted. Fungal pathogens had a negative effect on plant growth (leaf area ratio and relative growth rate of leaves) and on midday water potential (Ψ_{MD}). Our findings

also show that whole plant photosynthesis was greatly reduced in inoculated trees as water stress increased. These results reveal that whole plant photosynthesis is severely affected by the interaction of fungal pathogens and water stress intensity. In addition, the degree of virulence of each pathogen is not exacerbated by water stress conditions. Our synthesis provides a framework for understanding walnut tree responses under water deficit irrigation.

Introduction

Increasingly erratic changes in rainfall patterns and reductions in precipitation can cause substantial negative impacts on plant productivity (Shashidhar et al. 2013; Brown et al. 2015). Frequent and severe droughts pose a major constraint to plant productivity with potentially detrimental morphological, physiological, and biochemical plant responses (Fahad et al. 2017). In arid and semi-arid regions, plants face a challenge for survival when water supply to the roots is limited or the loss of water through transpiration is high (Anjum et al., 2011). Although plant drought responses vary among species (Demirevska et al. 2009; Pivovaroff et al. 2016), all plant species experience increasing tension in their xylem with increasing drought, potentially limiting the transport of water to transpiring and photosynthesizing leaves, thus reducing carbon assimilation, and increasing the risk of hydraulic failure. However, one critical question that is growing in importance is the link between drought and resistance to diseases and pathogens (Agustí-Brisach et al. 2020). This is especially critical for xylem-dwelling pathogens that may experience increased advantages of weakened plants during drought, and inhabit the water transport system, thus worsening plant water status directly (Edwards et al. 2007; Knipfer et al. 2018). In this

study, we address the interactions of drought and xylem- dwelling pathogens on the physiological performance, growth, and yield of a perennial crop plant.

Frequent drought events along with compromised water availability and quality and limiting arable land have caused tremendous problems in agricultural ecosystems (Luo et al., 2019). To close the gap of maximizing crop yield under optimal conditions, reduce the use of pesticide and fertilizer, and ensure world food security, there is the need to breed water saving and drought resistant crops. In the past 50 years, the development of breeding programs has focused on genetic engineering, biotechnology, propagation, nutrition, and production techniques (Bernard et al., 2018). The purpose is to make breeding more efficient along with the application of new molecular and genomic methods for crop improvement. The ultimate goal is to increase yield and quality, select leafing and fruiting date for early harvest dates, and increase resistance to diseases and abiotic stress (Bernard et al., 2018). Despite the breeding improvements, it still takes years to produce an improved variety making it difficult to keep up with the growing pace of demand for new improved crops (Acquaah, 2012). Furthermore, although growers would prefer to use crops with shorter reproductive cycles which could allow faster production, the release of new plant varieties still need to be adapted to local environmental conditions, as well as to evaluate for their performance in extreme environmental conditions and their tolerance to current and future pest and diseases, in order to maintain high production and quality (*Kubota* et al., 2008). In addition, growers still need to improve and employ more efficient crop management specifically addressing the concepts of water, nutrition, soil, physiology, and

defense to mitigate the effects of both abiotic and biotic stress on crops (Bernard et al., 2018).

In many commercial crops, fungal vascular pathogens such as *Esca*, *Botryosphaeria* and *Eutypa* dieback are the causal agents of limiting crop productivity. It has been reported that the prevalence of these pathogens in orchards is 40% in almonds (Olmo, 2016), 70-90% in mature olives (Moral et al., 2010) and could result in 100% crop loss as reported in some pistachio orchards (Michailides, 1991). Furthermore, it is reported that when the host is predisposed to drought the plant-pathogen interaction is influenced positively or negatively thereby exacerbating or diminishing the severity of the disease (Slippers and Wingfield 2007; Chojak et al., 2018). These vascular pathogens are described as saprophytes or endophytes (latent pathogens) causing diseases such as canker, fruit blight and dieback. Because these pathogens remain active for many years in the host, the extent of yield losses in many crops is still uncertain (Moral et al., 2019).

Specific vascular pathogens affect annual and perennial crops by interfering with the vascular system composed of xylem vessels, tracheid elements, and phloem elements. The xylem conducts water from roots to the stem and provides mechanical support to the canopy. When vascular pathogens invade the xylem, they cause death in a portion of the vascular cambium (Pearce 1996). In response to pathogen attack, the plant produces tyloses which leads to the blockage of vessels causing loss of xylem function (Pouzoulet et al., 2014). Furthermore, pathogen infection also results in the entry of air into the xylem-network (McElrone et al., 2018). These conditions disrupt the transport and distribution of water and nutrients to the plant. Studies in grapevine demonstrated that Dutch elm vascular

disease induced embolism in vessels and the infection interfered with xylem function (Edwards et al., 2007). In other cases, Petri disease has been associated with drought stress and the infection causes a substantial loss of trans-sectional area of sap-conductive xylem. The disease infection and the embolism caused by drought stress disrupts water transport and extends to non-symptomatic areas of the xylem (Edwards et al., 2007).

Several studies have focused on an individual factor: (i) the impact of drought on the physiological responses of trees, or (ii) biotic factors and the effects on tree health. However, it is still unclear how drought and pathogen interactions negatively affect physiological functions of walnut trees. Therefore, we need a more integrated approach to understand the relationship between drought and fungal attack on walnut tree physiology performance that will lead to an improved crop management. We hypothesize that (a) water stress treatments increase susceptibility to canker disease, (b) cankers negatively impact walnut physiological performance, and (c) the degree of severity varies across water regimes. To test this hypothesis, we characterized water relations and two different fungal interactions (*Diplodia mutila* and *Neofusicoccum parvum*) under three water deficit irrigation treatments. Our aims were: (i) to investigate the effects of water deficit × pathogen interaction on physiological and morphological traits in young walnut trees, (ii) to compare the effect of *Botryosphaeria* species on water potential across the different water deficit irrigation treatments, and (iii) to determine whether different water deficit treatments influence the development of canker in young walnut trees inoculated by both *Botryosphaeria* species.

Materials and Methods

The study consisted of a completely randomized design experiment conducted in a lath house at University of California Riverside (UCR) to study the effect of water deficit irrigation on young walnut trees inoculated with *N. parvum* and *D. mutila*. Ninety-2-year-old "paradox" walnuts (*Juglans regia*) from Duarte nursery were planted into 9 L pots filled with clay loam soil (UCR Soil Mixture) (Icks, Cott, 2011). Walnut trees were divided into nine groups (10 plants/group) for three different water regimes and each group received the following treatment: (1) – water stress (100% water holding at field capacity), - inoculation (Control); (2) – water stress (100% water holding capacity), + *N. parvum*; (3) – water stress (100% water holding capacity), + *D. mutila*; (4) + water stress (25% deficit irrigation), - inoculation; (5) + water stress (25% deficit irrigation), + *N. parvum*; (6) + water stress (25% deficit irrigation), + *D. mutila*; (7) + water stress (75% deficit irrigation), - inoculation; (8) + water stress (75% deficit irrigation), + *N. parvum*; (9) + water stress (25% deficit irrigation), + *D. mutila*.

To induce water stress, irrigation was restricted for groups 4-9 from February to July 2020. Water restriction was estimated by water loss after weighing 20 additional walnut plants in pots that have the same characteristics of cultivar and pot condition, and then watering all plants in the stress groups with a volume equivalent to the mean water loss (Icks et al. 2011). In addition, soil water content was measured using a soil moisture probe once a week to estimate water content in the soil.

Culture and inoculation of walnut branches with Botryosphaeriaceae.

Pure cultures of both pathogens *N. parvum* and *D. mutila* were grown on potato dextrose agar (PDA) media at 25°C for five days under normal light for 12 h each day (Mora et al. 2014). In February 2020, a wound 5 mm in diameter was made on the stem approximately 5 cm above ground level using a cork borer to inoculate walnut trees with mycelium plugs of *N. parvum* and *D. mutila*. Control plants were inoculated with sterile agar mycelium plugs. Upon insertion of mycelium plugs, each wound was sealed with parafilm and evaluated for canker development by July 2020.

Gas exchange measurements.

Three-years-old *J. regia* plants were selected to measure photosynthetic rate (*A*), transpiration (*E*), stomatal conductance (G_s) and internal CO_2 (C_i) using a portable Li-Cor LI-6800 (using reference CO_2 at 400 ppm, fan speed at 8,000 rpm, temperature 25°*C* and ambient light) (Li-Cor, Inc. Lincoln, NE, USA) device. The measurements for gas exchange, water potential and chlorophyll fluorescence were performed on the same leaf. The measurements were conducted once per week between 0700 and 11-00 h from May to July 2020 for all treatments. Five trees were selected randomly from each group and three fully developed leaves selected for each plant to measure gas exchange. Chlorophyll fluorescence (F_v/F_m) was measured on darkened leaf samples between midnight and 0100 h on a single leaf for all five random trees in each group.

Water potential measurements.

Water potential at predawn (Ψ_{PD}) and midday (Ψ_{M}) was conducted between May and July using Scholander Pressure Chamber (Deloire et al. 2011). Every two-weeks,

predawn water potential was conducted between 0200-0500 h prior to sunrise and mid-day between 1100-1300 h. The measurements were conducted on four random plants in each group for all treatments. For measurements, two fully developed random leaves in the upper canopy were foiled and covered with Whirl-Pak bags 8 hours prior to measurement to allow tissue to equilibrate with stem xylem. Leaves were excised from the petiole using a razor blade. Subsequently, using the razor blade, 1 cm of the leaf lamina was cut to insert the midvein into the pressure chamber seals. The chamber was pressurized slowly, and pressure was recorded when a water meniscus began to form on the cut petiole surface (Knipfer et al., 2018). Furthermore, delta was calculated from the difference between mid-day and pre-dawn water potential values and recorded.

Whole plant hydraulic conductance.

Plant hydraulic conductance (K_{plant}) was calculated from the relationship between single leaf transpiration and soil-leaf water potential. According to Breda et al. (1995), soil water potential is close to predawn water potential. Therefore, K_{plant} can be estimated from single leaf transpiration (E) rate and difference between Ψ_{PD} and Ψ_{leaf} using the following formula: $K_{plant} = E/(\Psi_{PD} - \Psi_{leaf})$ (Sperry and Pockman 1993). The measurements of E, Ψ_{PD} and Ψ_{leaf} were conducted as previously described.

Morphological measurements and dry weights.

For all trees, stem diameter (mm) was measured 3 cm above ground using a Vernier caliper. Stem height (cm) was also measured from the soil surface to the apical meristem using tape a measure. Both measurements were conducted once a month between 0800 and 1000 h. The total amount of leaves for every plant was also recorded towards the end of

the experiment. Leaf area (cm^2) was calculated by using a leaf area meter (Li 3000A, Li-Cor). To determine biomass (g), four walnut trees from each group were selected. All leaves were removed from the stem using pruning shears. The leaves and stems were immediately wrapped in foil and placed in an oven for 48h at 65°C (Santiago et al., 2000). The root system was removed from the pot, placed in a stainless sieve, and washed to remove adherent soil. After the root system became dry, it was placed in foil and dried out for 48 h at 65°C.

Using a digital balance, dry weight (g) for leaves, stems and roots were measured and recorded. Specific Leaf area (*SLA*) was calculated as leaf area per leaf mass (cm^2/g) excluding the petiole. Leaf area ratio (*LAR*) was calculated as total plant leaf area per total plant biomass (cm^2 leaf/g plant). Root to shoot ratio (*R/S*) was calculated as total below-ground mass per total above-ground mass (g/g plant) (Santiago et al., 2012). Relative growth rate of height (RGR_H; cm/cm × month) was calculated as $(\ln H_1 - \ln H_0)/(t_1 - t_0)$ where H_0 and H_1 were initial and final heights (cm) and t_1-t_0 was the time period (months). A similar equation to estimate relative growth rate of leaf count (RGR_L). Total plant leaf area (m^2/plant) was calculated by multiplying leaf area (m^2) × number of leaves/plants. The instantaneous whole plant photosynthesis was calculated by multiplying photosynthetic rate (μmol $CO_2/m^2/s$) by total plant leaf area (m^2/plant).

Canker development and Koch's postulate.

By the end of the experiment, canker length was measured for all plants. Using a ruler, we peeled off portion of the stem and canker was measured upward and downward from the point of inoculation. To complete Koch's postulate, three plants from each group

for a total of 9 plants for each treatment (n=27) were selected randomly and necrotic wood pieces were collected close to the inoculation site. The necrotic wood was sterilized in 1% hypochlorite for 30 sec and washed in water 1 min three times, grown in PDA and incubated as previously described to determine the presence of the pathogen (Chen et al. 2014).

Statistical analysis.

Data was analyzed using R studio software 3.4.1 (RStudio, 2020) where water regime and inoculation were treated as independent factors. We performed a repeated measures two-way ANOVA for gas exchange, chlorophyll fluorescence, predawn and midday water potential, delta, hydraulic conductance measurements and for growth responses. Within-individuals effects evaluated variation among months and interactions among pathogens, water treatments and date. A one-way ANOVA followed by post-hoc Tukey HSD was also performed for the last date for each variable mentioned above including whole plant photosynthesis and canker length.

Results

There were no significant effects of pathogen on photosynthetic gas exchange (A, E, G_s), or chlorophyll fluorescence (Figure 3.1). However, water deficit irrigation had a significant effect on all gas exchange measurements and F_v/F_m (Figure 3.1) over time ($P<$ 0.05; Table S3.1). A stronger significant effect of water deficit irrigation was observed for A, E, G_s (P <0.001; Table 3.1) in the last date of the experiment. Statistically significant differences in predawn (not shown) and midday (Figure 3.2) water potential were observed between inoculated plants and the control (P<0.05) during the experiment (Table S3.1) and

the last date (Table 3.1). Water regimes also influenced on whole-plant hydraulic conductance (Table S3.1) (P<0.001). The difference between predawn and midday water potential (Delta) showed no significant differences in interactions between water and inoculation treatments, although it did vary significantly among water regimes (Table S3.1).

Through time both water deficit irrigation and fungal species did not have any effect on stem height (P>0.05) (Table S3.1). Water deficit irrigation treatments only had an effect on relative growth rate of leaves (Figure 3.4 B), diameter, stem dry mass, leaf dry mass, root dry mass and total dry mass (P<0.05) (Table S3.1). Total plant leaf area (m^2/plant) was influenced by pathogen treatments and water regimes (Figure 3.3 B). Pathogens reduced the area of leaves that could be supported by the plant, but not the gas exchange rates of individual leaves (Table S3.1, Figure 3.4). Walnut trees with 100% water content had a higher total plant leaf area as compared to the trees in the 75% and 25% water content (P<0.05) (Table S3.1). Water deficit irrigation had an impact on specific leaf area (*SLA*) and root/shoot ratio (P<0.05) (Table S3.1). Furthermore, leaf area ratio *LAR* (P<0.05) (Table S3.1, Figure 3.4 A) was negatively impacted by the water deficit × pathogen interaction where well-watered trees had the greatest leaf area followed by the 75% and 25% water deficit irrigations. Therefore, leaf area plays a critical role in controlling the water used by young walnut trees.

The one-way ANOVA for the last date of the experiment showed both water regimes and the treatments had a statistically significant effect on whole plant photosynthesis (P<0.001) (Figure 3.3 A). Walnut trees holding 100% water content

showed a greater carbon gain when compared to trees under 75% and 25% water content (Table 3.1). Lastly, canker size grew significantly larger in inoculated plants, compared to the control in all treatments (P<0.05) (Table S3.1).

Discussion

Water deficit caused a reduction in photosynthesis (A), transpiration (E), stomatal conductance (G_s), and chlorophyll fluorescence (F_v/F_m) in young walnut trees. It is important to remark that the negative impact on physiological traits is attributed to water deficit and not as a consequence of fungal infection by both *Botryosphaeria* species. In addition, morphological traits (relative growth rates) were also severely impacted by the irrigation regimes. However, there was a significant difference in root to shoot ratio in young walnut trees inoculated with *N. parvum* vs. *D. mutila*.

Our observations confirmed the effect of water deficit on transpiration rate as a response to low midday leaf water potential showing the minimum between -0.20 MPa and -3 MPa. When inoculated young walnut trees are exposed to water stress, transpiration rates decrease to prevent leaf dehydration and stomata are expected to close once leaf water potential reaches -1.6 MPa (Cochard et al. 2002). As canker develops, inoculum builds up under negative water potential, trees induce the release of tyloses causing clogging of the conductive tissue leading to a significant effect on water transport.

Cankers developed in the stem of all inoculated trees irrespective of water deficit regime. However, only under well-watered conditions did the development of cankers influence on photosynthetic carbon gain at the whole plant scale, which could be associated with loss of hydraulic conductivity and stomatal conductance. Overall, there was no

evidence of water deficit exacerbating canker development. In 2018, a study reported that higher relative canker growth rate is experienced in pre-inoculated droughted *Corymbia calophylla* plants (Hossain et al. 2018). It is possible that predisposing young walnut trees to drought stress before inoculation will make the trees more vulnerable to disease than well-watered trees. It is demonstrated that when the host is exposed to pathogen infection, the host releases secondary metabolites such as polyphenols to prevent further infection (Eyles et al. 2010). However, exposing the host to drought before pathogen infection may cause a reduction in biochemical defense thus inciting faster canker growth (Hossain et al. 2018).

Whole plant photosynthesis was greatly reduced as a consequence of the pathogen × water interaction. As trees were exposed to water stress, partial stomatal closure was induced to prevent hydraulic failure. Upon partial stomatal closure, photosynthesis decreases causing lower photosynthetic carbon uptake, and resulting in carbon depletion in sink tissues -leaves (Martínez-Vilalta 2014). It is possible that when lesion or necrosis expanded in the stem, carbohydrate transport was inhibited thus affecting sink tissues (leaf, stem, root). Our data also showed that there was no effect of pathogens on whole-plant hydraulic conductivity, suggesting that canker pathogens do not induce embolism in early stages of infection. However, *Botryosphaeria* species have been reported to be necrotrophic and endophytic organisms (Inderbitzin et al. 2010) and adding water stress could exacerbate damage leading to hydraulic failure and severe carbon startvation. As both pathogens destroy functional tissue in leaves, stem and roots, the host is forced to invest C in repair, which can cause an increase of C need from the host maintaining the

interaction for years until the host dies (Cherubini et al. 2002). Nonetheless, prolonged drought events will also reduce C reserves necessary for plant defense causing a depletion of resources required for repairing infected tissue or making the host run out of C at faster rate (Oliva et al. 2014).

On a global scale climate change has greatly affected agricultural activities making areas unsuitable for cultivation of crops (Brown et al. 2015). Some of the consequences of climate change are erratic patterns of rainfall, frequent heat waves, desertification, changes in flowering of fruit trees and early bud break for many woody species (Hassankhah et al. 2017; Ladwig et al. 2019). The fluctuation of climate conditions and water availability could be a limiting factor for tree growth, increasing chances for disease severity in nut crops. It has been demonstrated that climate warming is likely to increase disease pressure thus affecting the phenology for a variety of tree species (Luedeling et al. 2010). In California the production of walnuts is concentrated in the Central Valley where the vast majority of walnut orchards are located in dry areas (Geisseler and Horwath, 2016). Since 2014, prolonged events of extreme and exceptional drought have occurred more frequently, increasing evaporative demand and leading to a decline in walnut productivity and tree longevity (Drought.gov 2020). The early events of drought in the growing season are the most important limiting factors for growth and seedling development in walnuts since research findings reveal that seedlings are intolerant to drought (Gauthier 2011). The negative impact on growth also results in lower yield, lower kernel size and quality (Fulton and Buchner 2015).

The role of endophytic pathogens causing tree decline is still uncertain since their aggressiveness depends both on abiotic (e.g., environment) and biotic (e.g, plant-specific interaction) factors. Several research studies have documented that *Botryosphaeria* species remain latent from fall and winter until spring and summer in woody trees and become highly aggressive when the host is weakened as a consequence of different biotic or abiotic factors (López-Moral et al. 2020). For example, events of high rainfall followed by severe drought increases host susceptibility as reported in pistachio trees infected by *Neofusicoccum mediterraneum* (Ma et al. 2001).

In California ten species within the Botryosphaeriaceae have been reported including *Botryosphaeria dothidea*, *Diplodia mutila*, *D. seriata*, *Dothiorella iberica*, *Lasiodiplodia citricola*, *Neofusicoccum mediterraneum*, *N. nonquaesitum*, *N. parvum*, *N. vitifusiforme*, *Neoscytalidium dimidiatum*. They were all determined to be pathogenic and susceptible to the English walnut cultivars studied ('Chandler', 'Tulare', and 'Vina') (Chen et al., 2014). Among the ten species identified, *L. citricola* and *N. parvum* were described as the most virulent species.

To date, there are no reports of *Botryosphaeria* species specific to a particular host. For example, *Neofusicoccum parvum* has been reported to cause canker in other hosts including almonds in Spain (Agustí-Brisach et al. 2020) walnut in California, China, Italy (Chen et al. 2014; Yu et al. 2015; Gusella et al. 2020) and pistachio in California (Chen et al. 2014b). In the English walnut cultivars, *D. mutila* has been described as intermediate to weakly virulent and has been isolated from avocado and almond in California (Inderbitzin

et al., 2010), walnut and grapevine in California and Chile (Chen et al. 2014; Díaz et al. 2018; Úrbez-Torres et al. 2009; Besoain et al. 2017).

The degree of virulence varies among Botryosphaeria species and is not always correlated with isolation frequency. The pathogen's fitness depends on other factors such as adaptation to climatic conditions, resistance to fungicides, saprophytic phase and disease pressure originating from nearby alternate plant host (Moral et al. 2019; Jimenez Luna et al. *in press*.). For example, *D. seriata*, considered a weak pathogen in nut crops in CA, was abundant when isolated from almond trees causing tree decline in Mallorca, Spain (Inderbitzin et al. 2010; Olmo et al. 2016).

In summary, our results demonstrate a complex interaction between water deficit and fungal pathogens. While neither pathogen had a negative effect on most of the physiological and morphological traits there was evidence of negative performance of young walnut trees under water deficit irrigation. We demonstrate how carbon gain is impacted by both pathogen and water potential under well-watered and water deficit conditions and their relationship with canopy leaf area and transpiration. As young walnut trees were exposed to drought after inoculation, photosynthetic carbon gain was significantly higher in well-watered trees. In conclusion, our results indicate that walnut photosynthetic carbon gain represents a primary and most important metabolic pathway of water × pathogen interaction during drought events. This study expands the current knowledge of the impact of water deficit in plant-pathogen interaction by incorporating both plant physiology and pathology responses in a single patho-system study.

References

Acquaah, G. 2012. Principles of plant genetics and breeding. Chichester, UK: Wiley-Blackwell.

Agustí-Brisach, C., Moldero, D., Raya, M., Lorite, I. J., Orgaz, F., Trapero, A. 2020. Water Stress Enhances the Progression of Branch Dieback and Almond Decline under Field Conditions. *Plants* (*Basel, Switzerland*) 9(9): 1213.

Anjum, S. A., Wang, L. C., Farooq, M., Hussain, M., Xue, L. L., Zou, C. M. 2011. Brassinolide application improves the drought tolerance in maize through modulation of enzymatic antioxidants and leaf gas exchange. *J. Agron. Crop Science* 197: 177–185. doi: 10.1111/j.1439-037X.2010.00459.x.

Bernard, A., Lheureux, F., Dirlewanger, E. 2018. Walnut: past and future of genetic improvement. *Tree Genetics & Genomes* 14: 1. https://doi.org/10.1007/s11295-017-1214-0.

Besoain, X., Guajardo, J., Camps, R. 2017. First Report of *Diplodia mutila* causing gummy canker in Araucaria araucana (Molina) K. Koch in Chile. *Plant Disease* 101. 10.1094/PDIS-12-16-1730-PDN.

Brown, M. E., Antle, J. M., Backlund, P., Carr, E. R., Easterling, W. E., Walsh, M. K. 2015. Climate change, global food security and the US food system. US Global Change Research Program; 146 pp.

Chen, S. F., Morgan, D. P., Hasey, J. K., Anderson, K., Michailides, T. J. 2014. Phylogeny, morphology, distribution, and pathogenicity of Botryosphaeriaceae and Diaportheaceae from English walnut in California. *Plant Disease* 98: 636-652.

Cherubini, P., Fontana, G., Rigling, D., Dobbertin, M., Brang, P., Innes, J. L. 2002. Tree-life history prior to death: two fungal root pathogens affect tree-ring growth differently. *Journal of Ecology* 90: 839–850.

Chojak-Koźniewska, J., Kuźniak, E., Zimny, J. 2018. The Effects of Combined Abiotic and Pathogen Stress in Plants: Insights from Salinity and *Pseudomonas syringae* pv *lachrymans* Interaction in Cucumber. *Frontiers in plant science* 9: 1691. https://doi.org/10.3389/fpls.2018.01691.

Cochard, H., Coll, L., Roux, X., Améglio, T. 2002. Unraveling the Effects of Plant Hydraulics on Stomatal Closure during Water Stress in Walnut. *Plant physiology* 128: 282-90. 10.1104/pp.010400.

Deloire, A., Heyns, D. 2011. The Leaf Water Potentials: Principles, Method and Thresholds. Wineland Magazine.

Demirevska, K., Zasheva, D., Dimitrov, R., Simova-Stoilova, L., Stamenova, M., Feller, U. 2009. Drought stress effects on Rubisco in wheat: changes in the Rubisco large subunit. *Acta Physiology Plant* 31: 1129–1138. doi: 10.1007/s11738-009-0331-2.

Díaz, G., Latorre, B., Ferrada, E., Gutierrez, M., Bravo, F., Lolas-Caneo, M. 2018. First Report of *Diplodia mutila* Causing Branch Dieback of English Walnut cv. Chandler in the Maule Region, Chile. *Plant Disease* 102. 10.1094/PDIS-11-17-1860-PDN.

Edwards, J., Pascoe, I. G., Salib, S. 2007. Impairment of Grapevine Xylem Function by Phaeomoniella chlamydospora Infection Is Due to More than Physical Blockage of Vessels with 'Goo'. *Phytopathologia Mediterranea* 46: 87-90. 10.14601/Phytopathol_Mediterr-1858.

Eyles, A., Bonello, P., Ganley, R., Mohammed, C. 2010. Induced resistance to pests and pathogens in trees. *The New phytologist* 185(4): 893–908. https://doi.org/10.1111/j.1469-8137.2009.03127.x.

Fahad, S., Bajwa, A. A., Nazir, U., Anjum, S. A., Farooq, A., Zohaib, A., Sadia, S., Nasim, W., Adkins, S., Saud, S., Ihsan, M. Z., Alharby, H., Wu, C., Wang, D., Huang, J. 2017. Crop Production under Drought and Heat Stress: Plant Responses and Management Options. *Frontiers in plant science* 8: 1147. https://doi.org/10.3389/fpls.2017.01147.

Hassankhah, A., Vahdati, K., Rahemi, M., Hassani, D., Sarikhani, K. S. 2017. Persian Walnut phenology: effect of chilling and heat requirements on budbreak and flowering date. *International Journal of Horticultural Science and Technology* 4(2): 259–271.

Hossain, M., Veneklaas, E., Hardy, G., Poot, P. 2018. Tree host-pathogen interactions as influenced by drought timing: linking physiological performance, biochemical defense and disease severity. *Tree physiology* 39. 10.1093/treephys/tpy113.

Gauthier, M. M., Douglass, J. 2011. Walnut (*Juglans* spp.) ecophysiology in response to environmental stresses and potential acclimation to climate change. *Annals of Forest Science* 68. 10.1007/s13595-011-0135-6.

Geisseler, D., Horwath, W. R. 2016. Walnut Production in California. CDFA. https://apps1.cdfa.ca.gov/FertilizerResearch/docs/Walnut_Production_CA.pdf.

Gusella, G., Giambra, S., Conigliaro, G., Burruano, S., Polizzi, G. 2020. Botryosphaeriaceae species causing canker and dieback of English walnut (Juglans regia) in Italy. *Forest Pathology* 51. 10.1111/efp.12661.

Icks, J. W., Cott, S. S. 2011. Sosnowsky Studies on the effect of water and temperature stress on Grapevine inoculated with Eutypa lata 50: 127–139.

Inderbitzin, P., Bostock, R., Trouillas, F. 2010. A six locus phylogeny reveals high species diversity in Botryosphaeriaceae from California almond. *Mycologia* 102: 1350-68. 10.3852/10-006.

Knipfer, T., Barrios-Masias F., Cuneo, I. F., Bouda, M., Albuquerque, C. P, Brodersen, C. R., Kluepfel, D. A., McElrone, A. J. 2018. Variations in xylem embolism susceptibility under drought between intact saplings of three walnut species. *Tree Physiology* 38:1180–1192.

Kubota, C., McClure, M., Kokalis-Burelle, N., Bausher, M., Rosskopf, E. 2008. Vegetable Grafting: History, Use, and Current Technology Status in North America. *HortScience*: a publication of the American Society for Horticultural Science 43. 10.21273/HORTSCI.43.6.1664.

Ladwig, L., Chandler, J., Guiden, P., Henn, J. 2019. Extreme winter warm event causes exceptionally early bud break for many woody species. Ecosphere. 10. e02542. 10.1002/ecs2.2542.

López-Moral, A., Lovera, M., Raya, M., Cortés-Cosano, N., Arquero, O., Trapero, A., Agustí-Brisach, C. 2020. Etiology of Branch Dieback and Shoot Blight of English Walnut Caused by Botryosphaeriaceae and *Diaporthe* Species in Southern Spain. *Plant disease* 104(2): 533–550. https://doi.org/10.1094/PDIS-03-19-0545-RE.

Luedeling, E., Steinmann, K., Zhang, M., Brown, P., Grant, J., Girvetz, E. 2010. Climate change effects on walnut pests in California. *Global Change Biology* 17: 228-238. 10.1111/j.1365-2486.2010.02227.x.

Luo, L., Xia, H., Lu, B. R. 2019. Editorial: Crop Breeding for Drought Resistance. *Frontiers in plant science* 10: 314. https://doi.org/10.3389/fpls.2019.00314.

Ma, Z., Morgan, D., Michailides, T. 2001. Effects of water stress on Botryosphaeria blight of pistachio caused by *Botryosphaeria dothidea*. *Plant Disease* 85: 745–749. https://doi.org/10.1094/PDIS.2001.85.7.745.

Martinez-Vilalta, J. 2014. Carbon storage in trees: pathogens have their say. *Tree Physiol* 34: 215–217.

McElrone, A. J., Jackson, S., Habdas, P. 2008. Hydraulic disruption and passive migration by a bacterial pathogen in oak tree xylem. *Journal of Experimental Botany* 59: 2649–2657.

Michailides, T. J. 1991. Pathogenicity, Distribution, Sources of Inoculum, and Infection Courts of *Botryosphaeria dothidea* on Pistachio. *Phytopathology* 81(5): 566. https://doi.org/10.1094/Phyto-81-566.

Moral, J., Morgan, D., Trapero-Casas, A. 2019. Ecology and Epidemiology of Diseases of Nut Crops and Olives Caused by Botryosphaeriaceae Fungi in California and Spain. *Plant Disease*. 10.1094/PDIS-03-19-0622-FE.

Moral, J., Muñoz-Díez, C., González, N., Trapero, A., Michailides, T. J. 2010. Characterization and pathogenicity of Botryosphaeriaceae species collected from olive and other hosts in Spain and California. *Phytopathology* 100: 1340-1351.

Oliva, J., Stenlid, J., Martinez, V. J. 2014. The effect of fungal pathogens on the water and carbon economy of trees: Implications for drought-induced mortality. *New Phytologist* 203. 10.1111/nph.12857.

Olmo, D., Armengol, J., León, M., Gramaje, D. 2016. Characterization and pathogenicity of Botryosphaeriaceae species isolated from almond trees on the island of Mallorca (Spain). *Plant Disease* 100:2483-2491. https://doi.org/10.1094/PDIS-05-16-0676-RE.

Pearce R. B. 1996. Antimicrobial defences in the wood of living trees. *New Phytologist* 132: 203–233 10.1111/j.1469-8137.1996.tb01842.x

Pivovaroff AL, Pasquini SC, De Guzman ME, Alstad KP, Stemke J, Santiago LS (2016) Multiple strategies for drought survival among woody plant species. *Functional Ecology* 30: 517–526.

Pouzoulet, J., Jacques, A., Besson, X., Dayde, J., Mailhac, N. 2013. Histopathological study of response of *Vitis vinifera* cv. Cabernet Sauvignon to bark and wood injury with and without inoculation by Phaeomoniella chlamydospora. *Phytopathologia Mediterranea* 52: 313-323. 10.14601/Phytopathol_Mediterr-12581.

Santiago, L., Wright, S. J., Harms, K., Yavitt, J., Korine, C., Garcia, M., Turner, B. 2012. Tropical tree seedling growth responses to nitrogen, phosphorus and potassium addition. *Journal of Ecology* 100: 309 - 316. 10.1111/j.1365-2745.2011.01904.x.

Santiago, L., Lau, T. S., Melcher, P., Steele, O., Goldstein, G. 2000. Morphological and Physiological Responses of Hawaiian Hibiscus tiliaceus Populations to Light and Salinity. *International journal of plant sciences* 161: 99-106. 10.1086/314236.

Shashidhar, H., Kanbar, A., Toorchi, M., Malleshachar, R., Kundur, P., Gowda, V., Soman, R., Kumar, N., Bekele, B., Puttaswamygowda, B. 2013. Breeding for Drought Resistance Using Whole Plant Architecture — Conventional and Molecular Approach. 10.5772/54983.

Slippers, B., Smit W. A., Crous, P. W., Countinho, T. A., Wingfield, B. D., Wingfield, M. J. 2007. Taxonomy, phylogeny and identification of Botryosphaeriaceae associated with pome and stone fruit trees in South Africa and other regions of the world. *Plant Pathology* 56: 128–139.

Sperry, J. S., Pockman, W. T. 1993. Limitation of transpiration by hydraulic conductance and xylem cavitation in *Betula occidentalis*. *Plant, Cell & Environment* 16: 279–287. doi:10.1111/j.1365-3040.1993.tb00870.x.

Úrbez-Torres, J. R., Gubler, Walter. 2009. Pathogenicity of Botryosphaeriaceae species isolated from grapevine cankers in California. *Plant Disease* 93: 584-592. 10.1094/PDIS-93-6-0584.

Yu, Z., Tang, G., Peng, S., Chen, H., Zhai, M. 2015. *Neofusicoccum parvum* causing canker of seedlings of Juglans regia in China. *Journal of Forestry Research* 26: 1019-1024. 10.1007/s11676-015-0130-0.

Table

Table 3.1 Results of one-way ANOVA for plant water relations and physiological traits on *Juglans* under 100%, 75%, 25% water content inoculated with *D. mutila* and *N. parvum* at week 11 (July 2020). Bold stars indicate statistically significant differences among means ($P < 0.05$).

Parameters		Inoculation			Water			Inoculation × water		
	df (resid)	*F*	*P*	sig.	*F*	*P*	sig.	*F*	*P*	sig.
Photosynthesis (*A*)	126	1.7	1.95E-01	-	64.4	2.00E-16	✶✶✶	0.94	4.45E-01	-
Transpiration (*E*)	126	0.4	6.54E-01	-	91.4	2.00E-16	✶✶✶	0.60	6.66E-01	-
Stomatal conductance (*Gs*)	126	0.4	6.82E-01	-	109.3	2.00E-16	✶✶✶	0.57	6.84E-01	-
Chlorophyll fluorescence (*Fv*)	36	1.8	1.78E-01	-	5.8	6.75E-03	✶✶	1.01	4.15E-01	-
Predawn water potential	27	2.1	1.39E-01	-	2.3	1.20E-01	-	0.47	7.55E-01	-
Midday water potential	30	12.2	1.69E-04	✶✶✶	18.2	9.98E-06	✶✶✶	1.33	2.84E-01	-
Delta water potential	30	5.1	1.35E-02	✶	7.6	2.44E-03	✶✶	1.74	1.71E-01	-
Hydraulic conductivity (*Kh*)	27	2.6	9.57E-02	-	4.3	2.31E-02	✶	2.12	1.06E-01	-
Whole Plant Photosynthesis (µmolCO2/plant/s	27	11.3	2.79E-04	✶✶✶	76.1	7.99E-12	✶✶✶	3.10	3.19E-02	✶

Figures

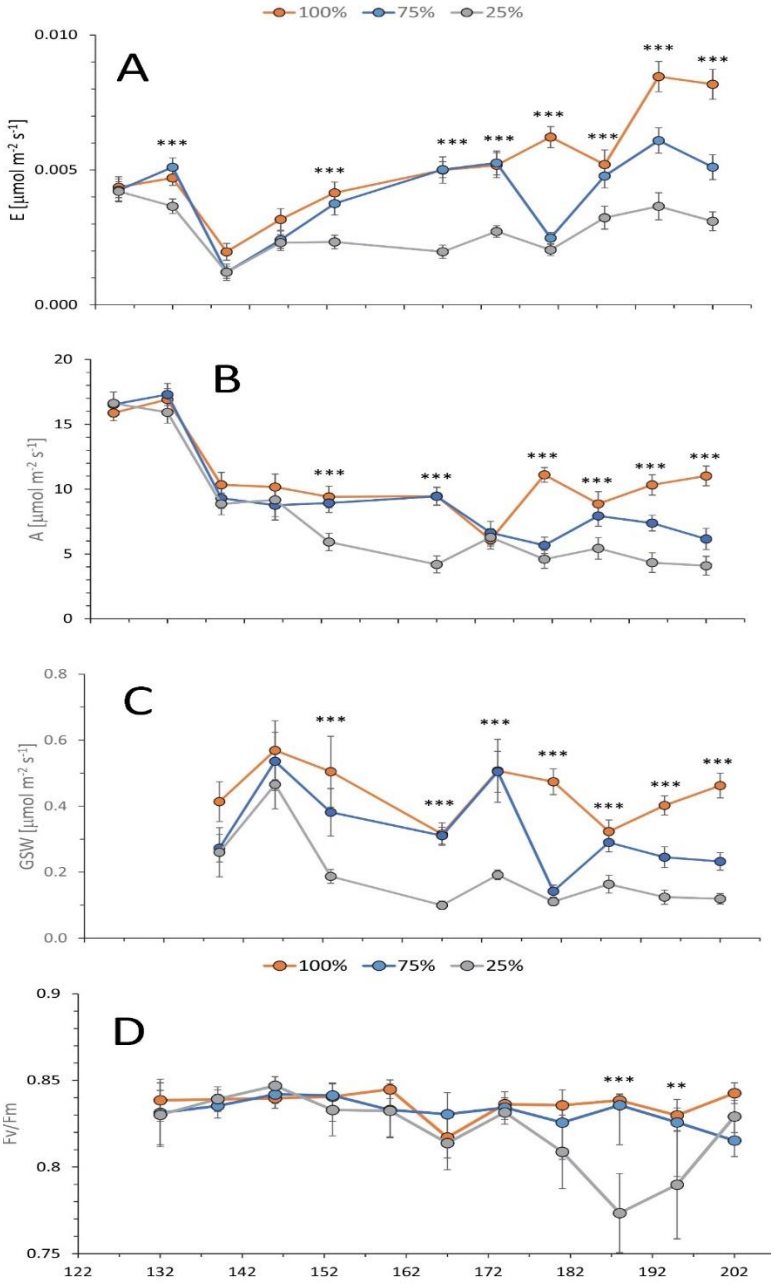


Figure 3.1 Measurements of transpiration (**A**), photosynthesis (**B**), stomatal conductance (**C**) and chlorophyll fluorescence (D). Data were collected for all three irrigation treatments: 100% (●), 75% (●), 25% (●) water content for 11 weeks. Each point is the mean ± SE of n= 5 trees. Stars on top show level of significance (P<0.05).

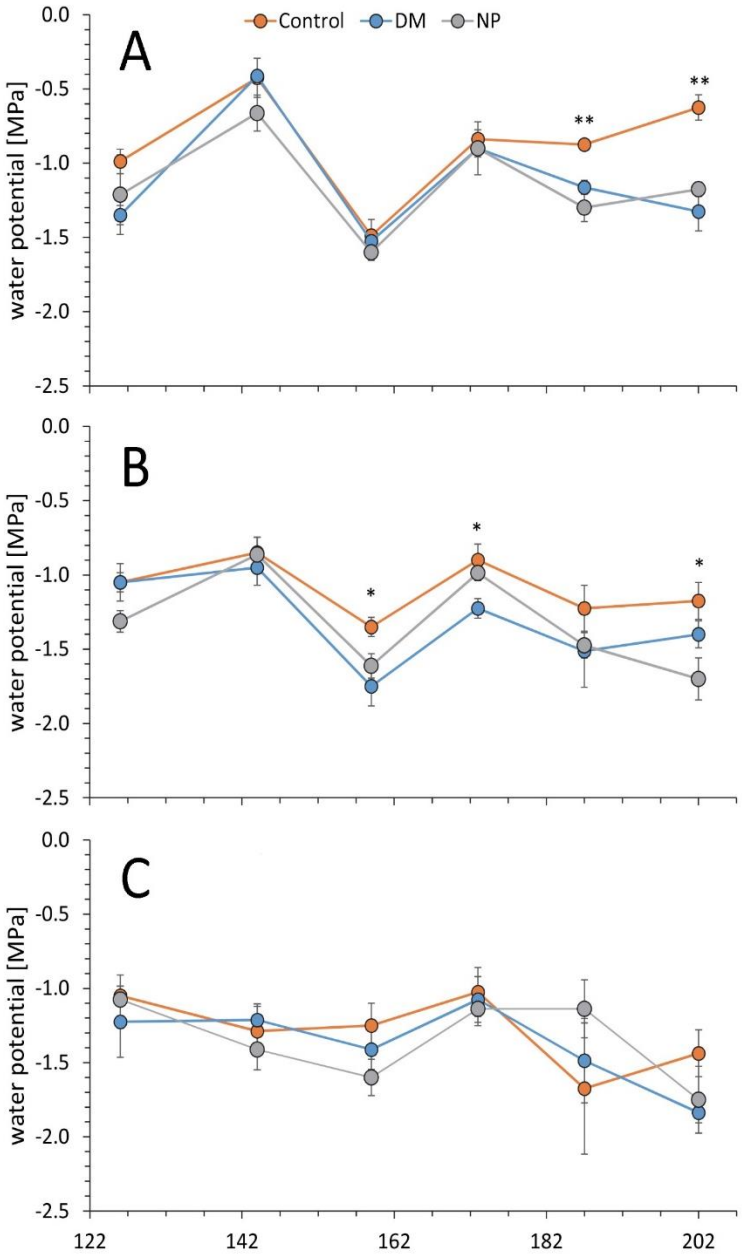
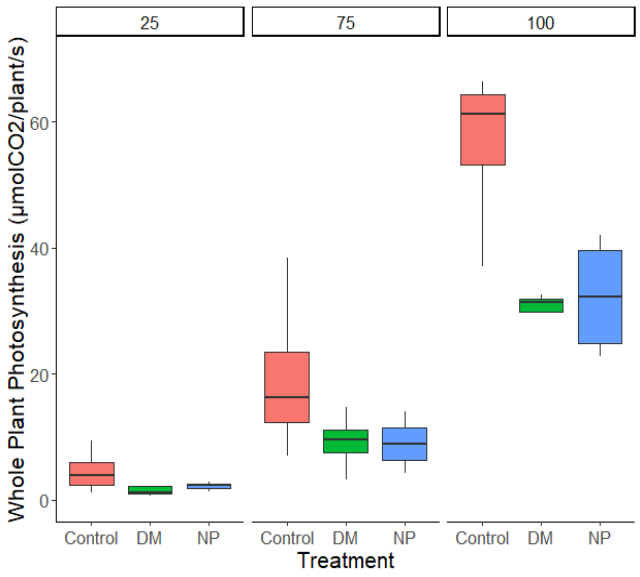


Figure 3.2 Midday leaf water potential (Ψ_{MD}) measurements for *J. regia* starting May to July 2020. 100% (**A**), 75% (**B**) and 25% (**C**) water content, control (●) *Diplodia mutila* (●) and *Neofusicoccum* parvum (●). Data were collected for all nine treatments (water × interaction) after 11 weeks. Stars on top show level of significance (P<0.05). Each point is the mean ± SE of n= 4 trees/group.

A



B

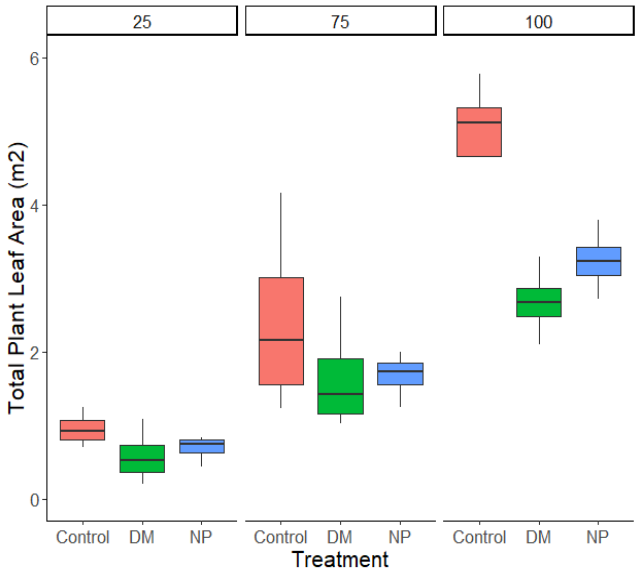
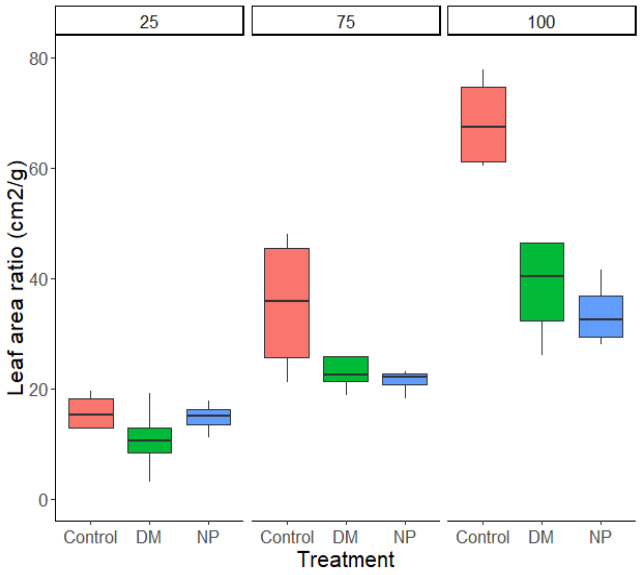


Figure 3.3 Box and whisker charts showing variation of Total plant leaf area (m2) (**A**) and carbon gain (umolCO2/plant/s) (**B**) for both *N. parvum* and *D. mutila* and the control under 100%, 75% and 25% water treatments: (**A**) showing a reduction in leaf area when water supply is a limiting factor and, (**B**) illustrating carbon gain increase in the control when water is available at 100%.

A



B

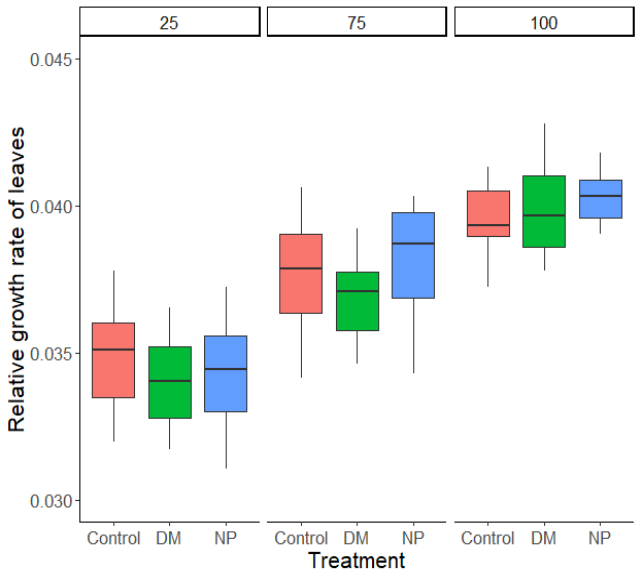


Figure 3.4 Box and whisker charts showing variation of (**A**) Leaf area ratio (cm2/g), and (**B**) relative growth rate of leaves impacted by *N. parvum* and *D. mutila* and the control under 100%, 75% and 25% water treatments. (**A**) showing a reduction in leaf area ratio when water supply is a limiting factor and the presence of fungal pathogens, (**B**) number of leaves increase as water becomes available at 100%.

Supplemental Table

Table S3.1 Results of repeated-measures analysis of variance for plant water relations, physiological and morphological traits on *Juglans* under 100%, 75%, 25% water content inoculated with *D. mutila* and *N. parvum* after 11 weeks (May-July 2020). Bold stars indicate statistically significant differences among means ($P < 0.05$).

Parameters		Inoculation			Water			Inoculation × water		
	df (resid)	*F*	*P*	sig.	*F*	*P*	sig.	*F*	*P*	sig.
Photosynthesis (*A*)	9	0.78	4.88E-01	-	61.1	5.82E-06	✶✶✶	0.16	9.53E-01	-
Transpiration (*E*)	9	0.13	8.79E-01	-	76.2	2.29E-06	✶✶✶	0.65	6.44E-01	-
Stomatal conductance (*Gs*)	26	0.08	9.23E-01	-	72.0	2.52E-11	✶✶✶	0.34	8.47E-01	-
Chlorophyll fluorescence (*Fv*)	9	0.40	6.48E-01	-	7.5	1.20E-02	✶	0.70	6.10E-01	-
RGR_height	98	0.13	8.76E-01	-	2.1	1.27E-01	-	0.45	7.70E-01	-
RGR_leaves	98	1.60	2.07E-01	-	90.9	2.00E-16	✶✶✶	0.36	8.35E-01	-
RGR_diameter	99	1.37	2.58E-01	-	73.8	2.00E-16	✶✶✶	1.24	2.98E-01	-
RGR_stem dry weight	27	0.78	4.69E-01	-	5.7	8.35E-03	✶✶	1.07	3.93E-01	-
RGR_leaves dry weight	27	0.21	8.14E-01	-	38.3	1.31E-08	✶✶✶	1.43	2.50E-01	-
RGR_roots dry weight	27	1.66	2.09E-01	-	4.2	2.68E-02	✶	1.63	1.96E-01	-
Total dry mass	27	1.44	2.54E-01	-	8.1	1.77E-03	✶✶	1.71	1.78E-01	-
Total plant leaf area	27	9.19	9.06E-04	✶✶✶	55.4	2.77E-10	✶✶✶	2.18	9.81E-02	-
Specific leaf area (SLA)	27	0.18	8.38E-01	-	3.3	5.05E-02	✶	0.51	7.28E-01	-
root/shoot (R/S)	27	4.53	2.01E-02	✶	0.4	6.79E-01	-	0.87	4.95E-01	-
Leaf area ratio (LAR)	27	17.96	1.09E-05	✶✶✶	59.1	1.36E-10	✶✶✶	5.78	1.71E-03	✶✶
Predawn water potential	46	23.18	1.09E-07	✶✶✶	21.7	2.29E-07	✶✶✶	1.14	3.51E-01	-
Midday water potential	46	12.79	3.83E-05	✶✶✶	25.3	3.92E-08	✶✶✶	1.20	3.22E-01	-
Delta water potential	46	1.93	1.57E-01	-	5.7	5.92E-03	✶✶	0.78	5.46E-01	-
Hydraulic conductivity (*Kh*)	46	1.14	3.29E-01	-	9.1	4.35E-04	✶✶✶	0.52	7.19E-01	-
Canker length (mm)	99	12.37	1.61E-05	✶✶✶	0.5	6.04E-01	-	0.21	9.32E-01	-

General Conclusion

The current results provide insightful information about the etiology and epidemiology of wood diseases in almond and walnut production systems, especially for California and Chile. This dissertation verifies the presence that *Botryosphaeria* and *Diaporthe* species as cosmopolitan pathogens of almond and walnut trees in Californian and Chilean orchards. The taxonomy of each fungal species collected was confirmed morphologically and by DNA sequencing approaches. The construction of phylogenetic trees by sequencing several loci (ITS, EF, B-tub) allowed to confirm the identity of each pathogen underpinning the ecology and phylogenetic relationships among fungal species.

In addition, this work extended the knowledge on the epidemiology of those pathogens from our spore trapping data coupled with isolation of infected wood pieces. We report the existence and migration of five genera of *Botryosphaeria* and one genus of *Diaporthe* within the almond orchards and possibly the immigration of these pathogens from adjacent orchards. We also recovered insect-vectored *Ceratocystis* pathogen from almond trunk and scaffold.

Our findings also highlight how environmental factors such as host type, orchard age and wet events (rain and irrigation) affect pathogen profile. For example, several *Botryosphaeria* spp. were recovered from almond rather than walnut trees, and Botryosphaeriaceae incidence increased with orchard age. In contrast, more *Diaporthe* species were mostly recovered from mature-walnut trees. Our study also emphasizes that airborne pathogenic inoculum is higher during wet events vs. dry years.

Although, no spore trapping method was used in walnut trees in Chile, we do not discard the possibility that the immigration of fungal species also occurs from adjacent orchards (source of inoculum). Our work also supports that pruning should be done during dry weather since there is higher spore discharge during rainy seasons. It is recommended to use protective sprays to prevent further infection in wounds caused by pruning. The use of chemical management is necessary in orchards with high inoculum levels. Fungicides (Fluopyram + Tebuconazole) should be applied to wounded and infected areas during mid-May, mid-June, mid-July and following postharvest to minimize inoculum (Adaskaveg et al., 2017).

This study also investigates the effect of water stress × fungal interaction on walnut tree physiology and the potential efficacy of different water deficit irrigation treatments for further nut crop plantations in arid areas. All physiological traits were negatively affected by 75% and 25% water deficit irrigation. In particular, walnut trees under 75% water deficit showed lower A, E, and G_s. A reduction in water loss under drought conditions is achieved by transiently lowering G_s which aids in maintaining a lower leaf photosynthetic rate during water deficit. Our G_s data was also correlated with leaf water potential when trees were experienced water deficit. Lower water potential decreased G_s which then caused lower transpiration and lower photosynthetic rate. Our study shows that although trees were exposed to water deficit, water potential still helped continue photosynthesis and carbon gain.

Furthermore, although chlorophyll fluorescence (F_v/F_m) measurements are useful for identifying the severity of drought stress experienced by trees, our data shows that as

temperature increases toward the end of the experiment, water deficit had a minor effect on the quantum yield of photosystem II in trees exposed to 25% water deficit. Well-watered trees did not experience a lower F_v/F_m which indicates that photosynthesis, is not severely affected during the light period in the current study. The presence of both pathogens did not have any effect on leaf F_v/F_m since values rarely dropped below 0.78 even with trees that looked visually stressed. Although, we did not find chlorophyll fluorescence affected by the attack of fungal species, it is possible that affected trees by water stress support fewer leaves (lower total leaf mass) with more efficiently operating leaves. However, we have little evidence that chlorophyll fluorescence was damaged by fungal pathogens.

Most importantly, whole plant canopy photosynthetic rate was greatly reduced under full irrigation and pathogen interaction. This shows that whole-plant photosynthesis decline is more pronounced when attacked by a fungal pathogen but not when water stress deficit is a factor. Therefore, when canopy structure becomes reduced, the distribution of carbon declines leading to low fruit yield.

Wood canker affects tree canopy which can negatively impact tree growth. Our study showed that when water is the limiting factor, physiological and morphological traits are negatively impacted during the timeframe of our study. Trees experiencing lower water potential shed their leaves and suffered a reduction in stem. It is possible that severe drought may cause a greater effect on growth and mortality of the young walnut trees which can increase vulnerability to fungal disease severity due to small and shallow root system and limited carbohydrate reserved in leaves, stems, and roots.

Adopting integrated plant disease management is an effective strategy to reduce wood pathogen inoculum and improve yield production. In our studies we addressed disease epidemiology and etiology of fungal canker diseases. The information is useful for growers to prune their trees in the absence of rain events to avoid fungal spore discharge and reduce the probability of disease infection. We suspect that water stress can be a major predisposing factor of walnut to susceptibility by *Botryosphaeria* spp. The current information is useful to growers to manage their nut orchards properly and adopt the appropriate irrigation for successful nut production.

References

Adaskaveg, J.E., Buchner, R.P., Browne, G.T., Michailides, T.J., Hasey, J.K., Fichtner, E.J., Seybold, S.J., Bostock, R.M., Teviotdale, B.L. 2017. UC IPM Pest Management Guidelines: Walnut UC ANR Publication 3471. https://www2.ipm.ucanr.edu/agriculture/walnut/Botryosphaeria-Dieback/