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Comparative Profiling of Wood Canker Pathogens from Spore Traps and Symptomatic Plant Samples Within California Almond and Walnut Orchards

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Abstract

Fungi causing wood canker diseases are major factors limiting productivity and longevity of almond and walnut orchards. The goal of this study was to compare pathogen profiles from spore traps with those of plant samples collected from symptomatic almond and walnut trees and assess if profiles could be influenced by orchard type and age, rainfall amount and frequency, and/or neighboring trees. Three almond orchards and one walnut orchard with different characteristics were selected for this study. Fungal inoculum was captured weekly from nine trees per orchard using a passive spore-trapping device, during a 30-week period in the rainy season (October to April) and for two consecutive years. Fungal taxa identified from spore traps were compared with a collection of fungal isolates obtained from 61 symptomatic wood samples collected from the orchards. Using a culture-dependent approach coupled with molecular identification, we identified 18 known pathogenic species from 10 fungal genera (*Ceratocystis destructans*, *Collophorina hispanica*, *Cytospora eucalypti*, *Diaporthe ampelina*, *Diaporthe chamaeropis/rhusicola*, *Diaporthe eres*, *Diaporthe novem*, *Diplodia corticola*, *Diplodia mutila*, *Diplodia seriata*, *Dothiorella iberica*, *Dothiorella sarmentorum*, *Dothiorella viticola*, *Eutypa lata*, *Neofusicoccum mediterraneum*, *Neofusicoccum parvum*, *Neoscytalidium dimidiatum*, and *Pleurostoma richardsiae*), plus two unidentified *Cytospora* and *Diaporthe* species. However, only four species were identified with both methods (*Diplodia mutila*, *Diplodia seriata*, *Dothiorella Iberica*, and *E. lata*), albeit not consistently across orchards. Our results demonstrate a clear disparity between the two diagnostic methods and caution against using passive spore traps to predict disease risks. In particular, the spore trap approach failed to capture: insect-vectored pathogens such as *Ceratocystis destructans* that were often recovered from almond trunk and scaffold; *Diaporthe chamaeropis/rhusicola* commonly isolated from wood samples likely because *Diaporthe* species have a spatially restricted dispersal mechanism, as spores are exuded in a cirrus; and pathogenic species with low incidence in wood samples such as *P. richardsiae* and *Collophorina hispanica*. We propose that orchard inoculum is composed of both endemic taxa that are characterized by frequent and repeated trapping events from the same trees and isolated from plant samples, as well as immigrant taxa characterized by rare trapping events. We hypothesize that host type, orchard age, precipitation, and alternative hosts at the periphery of orchards are factors that could affect pathogen profile. We discuss the limitations and benefits of our methodology and experimental design to develop guidelines and prediction tools for fungal wood canker diseases in California orchards.

Keywords: almond, Botryosphaeriaceae, Diaporthaceae, epidemiology, *Juglans regia*, *Prunus dulcis*, spore trap, tree nuts, trees, walnut, wood canker

In 2018, the United States ranked first and second in almond (*Prunus dulcis*) and walnut (*Juglans regia*) production worldwide, respectively (U.S. Department of Agriculture-National Agricultural Statistics Service 2019). California accounts for >99% of the nation's production for those nut crops, with about 630,000 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; California Department of Food and Agriculture 2020). However, as these industries look to expand to meet the global demand, several factors are hindering those prospects. Among them, fungal wood canker diseases are impacting the longevity and productivity of perennial cropping systems. For example, these pathogens were responsible for a yield reduction ranging from 30 to 60% and 40 to 100% in California vineyards (Munkvold et al. 1994) and pistachio orchards (Moral et al. 2019b), respectively. In addition, wood canker diseases caused mortality of about 5% of walnut trees in nurseries (Chen et al. 2013a) and 25% of almond trees in young orchards (Chen et al. 2013b).

Band canker and Ceratocystis canker are common diseases of almond, and thousand cankers and Botryosphaeria canker are important diseases of walnut (Holland et al. 2019; Kolařík et al. 2011; Moral et al. 2019a, b). Cankers result in the loss of physiological functions of the host vascular system after the death of the cambium in response to fungal infection (Pearce 1996; Pouzoulet et al. 2022; Shigo and Marx 1977). When a canker encircles a major branch or a tree trunk, it girdles the bark entirely causing branch dieback or decline of the entire tree (Inderbitzin et al. 2010; Olmo et al. 2016). Additional wood canker symptoms 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 necrosis, and a ring of discoloration with brown to dark-brown vascular streaking (Holland et al. 2021; Lawrence et al. 2015; León et al. 2020; López-Moral et al. 2020). In almond, infection by wood canker pathogens is often accompanied externally by amber-colored balls of gum on the bark (Gramaje et al. 2012; Holland et al. 2021; Pouzoulet et al. 2022).

Wood canker diseases are caused by a complex of taxonomically unrelated fungal pathogens with broad host and geographical ranges, including several taxa in the Botryosphaeriaceae and Diaporthaceae families (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; Jiménez Luna et al. 2020; Lawrence et al. 2015; León et al. 2020; López-Moral et al. 2020; 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

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of wood canker diseases including *Ceratocystis*, *Collophorina*, *Cytospora*, *Eutypa*, *Geosmithia*, and *Phaeoacremonium* (Gramaje et al. 2012; Holland et al. 2018, 2021; Kolařík et al. 2011; Lawrence et al. 2018; Luo et al. 2020; Sohrabi et al. 2020; Trouillas and Gubler 2010). Many pathogens are airborne and water-splashed and infect open wounds of the plant host. 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; Trouillas et al. 2012; Úrbez-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 disease in almond and thousand cankers disease in walnut (Kolařík et al. 2011; Moller and DeVay 1968).

Wood canker diseases have become a growing concern among industry stakeholders, and their widespread occurrence in orchards is likely the result of a shift in cultural practices (Doll et al. 2013). Higher 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 shown in vineyards in 2021 (Henderson et al. 2021). More frequent 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 wood 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 canker pathogens to infect trees (Holland et al. 2021; Moral et al. 2019a, b).

The aim of this study was to qualitatively profile the pathogen inoculum present in spore traps of almond and walnut orchards and compare it to the pathogens isolated from symptomatic plant samples. We tested the hypothesis that these profiles yield comparable results, and that factors such as orchard type, age, climate, and location may influence those profiles. The results of this study improve our knowledge of the epidemiology of wood canker disease pathogens in almond and walnut and may contribute to the implementation of adapted management strategies.

Materials and Methods

Fungal culture and collection. We selected three almond orchards and one walnut orchard within a 30-km radius located in Merced County, California. The first almond orchard (Almond #1) was 10 years old at a planting density of 274 trees per ha (6.7 m row spacing × 5.4 m tree spacing) and located within 400 m of a riparian area, and had adjacent vineyard and walnut plantings. The three almond cultivars planted within this orchard were Carmel, Monterey, and Nonpareil grafted to the rootstock 'Bright's Hybrid'. The second orchard (Almond #2) was 6 years old at a planting density of 310 trees per ha (6.7 × 4.8 m) and had a history of wood canker disease. It was surrounded by other almond orchards and was planted with the cultivars Aldrich, Monterey, and Nonpareil grafted to the rootstock 'Viking'. The last almond orchard (Almond #3) was 4 years old at a planting density of 310 trees per ha (6.7 × 4.8 m), and composed of 'Aldrich', 'Carmel', and 'Nonpareil' grafted to 'Bright's Hybrid' rootstock; it was selected because of its proximity to the walnut orchard. The walnut orchard was a mature 15-year-old 'Tulare' walnut orchard grafted on 'Paradox' rootstock at a planting density of 178 trees per ha (8 × 7 m), and was planted directly adjacent to the Almond #3 orchard.

The spore-trapping method was adapted from Eskalen et al. (2013) and Úrbez-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-m height. In each orchard, traps were placed on nine trees in a 3 × 3 grid, spaced every five rows and every 10 trees within each row. Traps were collected weekly and replaced the same day for a total of 30 weeks from October through April, corresponding to the rainy season in California. Spore trapping was repeated for two consecutive rainy seasons for each orchard and the trials spanned the years 2012 to 2016 for all four orchards. To complement the spore trapping, we obtained total precipitation amount (mm) and number of rain events per week (numbers of days with at least 0.25 mm of rainfall in a single week) from two stations (Merced and Denair) of the California Irrigation Management Information System (CIMIS; https://cimis.water.ca.gov/) that were located near the commercial orchards where the experiments were conducted.

For spore-trap processing, each slide was placed inside a sterile 50-ml Falcon tube and mailed to the laboratory at the University of California-Riverside, where they were further processed. Slides were washed by adding 10 ml of a warm (~30°C) aqueous solution containing 0.5% Tween 20 to the Falcon tube and shaking it for 30 s. A 200-µl aliquot of this rinsate was cultured in duplicates on potato dextrose agar (PDA; BD Difco) culture medium amended with three antibiotics (ampicillin, neomycin, and tetracycline at 1 mg/liter each) to inhibit bacterial growth. Fungal isolates recovered from culturing were initially identified by morphology, and because of the high volume of cultures only a subsample of representative morphotype isolates were selected for molecular identification. Isolates in the Botryosphaeriaceae were selected using the dichotomous key for asexual morph (Phillips et al. 2013). *Diaporthe* isolates were selected by the production in culture medium of black conidiomata with alpha conidia (Udayanga et al. 2011).

For wood sample collection, 61 symptomatic wood samples from either the main structural framework of the tree (trunk and scaffold) or from the tree canopy (branch and twig) were collected from the four orchards and brought back to Kearney Agricultural Research and Extension Center and processed as described by Holland et al. (2021). Symptomatic wood samples were cut into small wood or bark pieces (approximately 3 × 3 × 3 mm) exposing the margin of infected and apparently healthy tissues, then surface-disinfested by submerging in 0.5% sodium hypochlorite for 2 min and 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 (i.e., 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 given above and incubated in a humid chamber as described in Holland et al. (2019). All culture plates were incubated at ambient conditions in the laboratory. After 7 days, fungal isolates were selected based on morphological characters and transferred to new PDA culture medium. All fungal isolates recovered from plant tissues were identified by DNA sequencing and comparison with reference sequences deposited in the GenBank database (Supplementary Table S1).

DNA extraction, PCR amplification, and DNA sequencing. DNA was extracted from fungal pure cultures recovered from both spore traps and plant samples 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 Thermocycler (Bio-Rad, Irvine, CA). Each reaction consisted of 17.4 µl of sterile H_2O, 2.5 µl of PCR buffer, 1 µl of dNTPs (10 mM each), 0.5 µl of each primer (4 µM), 2 µl of $MgCl_2$ (25 mM), and 0.1 µl of *Taq* DNA polymerase (5 U/µl), with DNA added at 1 µl (10 to 20 ng DNA/µl). The nuclear ribosomal internal transcribed spacer (ITS) region and the translation elongation factor 1α region (EF-1α) were amplified with the ITS1–ITS4 primer pair (White et al. 1990) and the EF1F–EF2R primer pair (Jacobs et al. 2004), respectively. 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 strand extension; the PCR was completed with a final 3-min extension at 7°C. Amplicons were run on a 1% agarose gel using gel electrophoresis at 110 V for 20 min and stained with Gel Red dye (Biotium Inc., Fremont, CA). PCR products were visualized as bands under UV light using a Gel Doc Imager (Bio-Rad, Irvine, CA) and amplicons were purified using a QiaQuick PCR Purification Kit (Qiagen, Valencia, CA). Forward and reverse reads were generated by Sanger sequencing performed at the University of California-Riverside Institute of Integrative Genome Biology sequencing core.

Phylogenetic analyses. Forward and reverse sequence reads were edited and combined into a consensus sequence using the tool

Sequencher v.5.0.1 (Gene Codes Corporation, Ann Arbor, MI). Sequences from each data partition (ITS and EF) were concatenated in the program Geneious v.6.0.6 (Biomatters Ltd., Auckland, New Zealand) and aligned using the ClustalW option followed by manual adjustments. Sequence alignments and phylogenetic analyses were performed separately for Botryosphaeriaceae and Diaporthaceae owing to their taxonomic divergence. The complete dataset of Botryosphaeriaceae consisted of 38 novel sequences and 23 reference sequences. The Diaporthaceae dataset consisted of 15 novel sequences and 30 reference sequences. Reference taxa were obtained from fungal culture collections, including the Westerdijk Institute/Centraalbureau voor Schimmelcultures (CBS-KNAW, Utrecht, The Netherlands), and the Culture Collection (CMW) of the Forestry and Agricultural Biotechnology Institute (University of Pretoria, South Africa). Their nucleotide sequences were downloaded from the GenBank sequence database maintained by NCBI and were included for alignment and phylogenetic analyses. GenBank accession numbers for all sequences are listed in Supplementary Table S1. Phylogenetic trees for the Botryosphaeriaceae and Diaporthaceae were constructed with the software MEGA X (Kumar et al. 2016; Stecher et al. 2020) using maximum-likelihood estimation 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.

Results

Total precipitation from October to April was below average for Merced County (approximately 278 mm CIMIS average per year for the 1999 to 2019 period) for 3 of the 4 years of the field trials (Table 1; Supplementary Table S2); these months are typically rainy months in California and have been shown previously to coincide with the occurrence of water-splash dispersed and airborne inoculum of fungal wood canker pathogens (Eskalen et al. 2013; Úrbez-Torres et al. 2010). In particular, 2013 to 2014 was an exceptionally dry year (~159 mm in total precipitation) with few rain events during the period of data recording (13 of the 30 weekly recording dates).

The pathogen profile from spore traps is presented at the genus level because only a representative subset of morphotype isolates for each genus was further selected for sequencing (Table 1). Our data provided evidence that spores of Diaporthaceae and Botryosphaeriaceae taxa were mainly released after rain, although *Diaporthe* and *Diplodia* spores were also trapped when rain events were not recorded by the CIMIS weather station (Supplementary Table S2). The data also suggested that total precipitation, number of rainy events, and orchard type and/or age could affect fungal spore-trapping frequency and profile (Table 1; Supplementary Table S2). For example, *Dothiorella* species were more frequently trapped in the older orchards and during wet years with respect to total precipitation and number of rain events (almond orchards #1 and #2), while almost no trapping of *Dothiorella* spores occurred in the youngest orchard even in a wet year (almond orchard #3). *Diaporthe* species were trapped 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* was the most widely dispersed taxon as it was trapped in all four orchards, albeit at a low frequency. *Cytospora*, *Eutypa*, *Neofusicoccum*, and *Neoscytalidium* were all specific to individual almond orchards and trapped at a low frequency.

For several representative morphotype isolates a single species was identified with >99% DNA sequence identity with CBS-KNAW type specimen isolates posted in the GenBank database, and thus no phylogenetic analyses were needed for identification. Those included *Ceratocystis destructans*, *Collophorina hispanica*, *Cytospora eucalypti*, *Eutypa lata*, and *Pleurostoma richardsiae*. In contrast, the subset of representative morphotype isolates for the Botryosphaeriaceae and Diaporthaceae groups collected from spore traps and tree samples showed DNA sequence variability, and phylogenetic analyses were used for species identification. Representative morphotype isolates for these two groups were pooled with type specimen isolates from CBS-KNAW and CMW (Supplementary Table S1). Alignment of 61 DNA sequences of the Botryosphaeriaceae resulted in a dataset of 1,182 nucleotide positions (494 positions in the ITS partition and 688 in the EF partition). These included 862 conserved sites (ITS = 377, EF = 485), 320 variable sites (ITS = 117, EF = 203), and 314 parsimony-informative sites (ITS = 114; EF = 200). The optimal

Table 1. Pathogen profile from spore traps within each orchard; data show the number of weekly trapping events and number of trees with repeated trapping events for each taxon; orchard characteristics are also shown including orchard type, year of the trial, total precipitation, and number of rain events from 1 October to 30 April

Orchard type	Orchard age (years)	Trial year	No. of rain events[a]	Total precipitation (mm)[a]	Fungal taxa cultured from traps[b]	No. of trapping events (weeks)[c]	No. of trees with repeated trapping events[d]
Almond #1	10	2012–2013	22	164.8	*Diaporthe*	1	–
					Dothiorella	11	4
					Neoscytalidium	1	–
		2013–2014	13	158.9	*Diplodia*	2	–
					Dothiorella	4	2
Almond #2	6	2013–2014	13	158.9	*Cytospora*	1	–
					Diplodia	1	–
					Dothiorella	3	1
		2014–2015	20	214.6	*Dothiorella*	9	1
					Eutypa	1	–
Almond #3	4	2014–2015	21	152	*Diaporthe*	1	–
		2015–2016	24	435.4	*Diaporthe*	2	–
					Diplodia	2	–
					Dothiorella	1	–
Walnut	15	2014–2015	21	152	*Diaporthe*	18	8
					Diplodia	3	2
					Neofusicoccum	3	1
		2015–2016	24	435.4	*Diaporthe*	17	9
					Diplodia	6	1
					Neofusicoccum	1	–
					Neoscytalidium	1	–

[a] Based on 30 weekly California Irrigation Management Information System reports from 1 October to 30 April each year (https://cimis.water.ca.gov/).
[b] Fungal taxa were named to the genus level based on initial morphological identification, and a subset of morphotype isolates was confirmed by internal transcribed spacer and translation elongation factor sequencing.
[c] Based on the 30 weekly trapping events from 1 October to 30 April, from any of the nine trees sampled.
[d] A dash (–) indicates fewer than two trapping events from the same tree (nine trees total).

model of nucleotide substitution inferred using the AIC was the Tamura–Nei model (Tamura and Nei 1993), with a discrete Gamma distribution and a proportion of invariant sites (TN93 + G + I). The tree with the highest log likelihood (−1,988.07) is shown in Figure 1. The alignment of 45 Diaporthaceae DNA sequences comprised 893 nucleotide positions (483 positions in the ITS partition and 410 in the EF partition), of which 411 were conserved (ITS = 324, EF = 87), 442 were variable (ITS = 155, EF = 287), and 382 parsimony-informative (ITS = 118, EF = 264). The AIC-inferred optimal model of nucleotide substitution was a general time reversible model (GTR; Nei and Kumar 2000) with a discrete Gamma distribution (G) and a proportion of invariant (I) sites (GTR + G + I). The tree with the highest log likelihood (−3,239.22) is shown in Figure 2.

Phylogenetic analyses of the combined ITS and EF sequences assigned the isolates collected from spore traps and plant samples of almond and walnut orchards to nine putative species belonging to four genera in the Botryosphaeriaceae family and five species in one genus in the Diaporthaceae family. Botryosphaeriaceae species included *Diplodia corticola*, *Diplodia mutila*, *Diplodia seriata*, *Dothiorella iberica*, *Dothiorella sarmentorum*, *Dothiorella viticola*, *Neofusicoccum mediterraneum*, *Neofusicoccum Parvum*, and *Neoscytalidium dimidiatum* (Fig. 1, Botryosphaeriaceae); and Diaporthaceae species included *Diaporthe ampelina*, *Diaporthe eres*, *Diaporthe novem*, and *Diaporthe rhusicola/chamaeropis*, plus an unidentified *Diaporthe* sp. (Fig. 2, Diaporthaceae). All the species clustered in clades that were supported with strong bootstrap values. Our phylogenetic reconstruction could not resolve the species boundaries between *Diaporthe chamaeropis* and *Diaporthe rhusicola* based only on the two DNA loci used. In addition, one isolate collected from a plant sample in the walnut orchard (WS3) clustered with *Diaporthe brasiliensis*, *Diaporthe caatingaensis*, and an unnamed CBS *Diaporthe* isolate and will be referred as *Diaporthe* sp. in this article.

The results of our diagnosis of symptomatic plant samples (Table 2) showed that the majority of trees were infected with *Diaporthe chamaeropis/rhusicola* (39%, 24 samples), although half of them came from the walnut orchard (12 of the 24 samples). The second most abundant taxon was *Ceratocystis destructans* (18%, 11 samples), and it was only found on the trunk and scaffold in almond orchards. *Diplodia seriata* (15%, nine samples) and *Diplodia mutila* (8%, five samples) were the most prevalent Botryosphaeriaceae fungi found on all almond tree parts. The remaining fungal taxa included, by decreasing order, *E. lata* (four samples); *Cytospora eucalypti*, *Dothiorella iberica*, and *Diaporthe novem* (two samples each); and *Collophorina hispanica*, *P. richardsiae*, and a *Diaporthe* sp. (one sample each). All three *Diaporthe* species isolated from plant samples came from the canopy (branch or twig) of almond and walnut trees.

In total, we identified from both spore-trapping and wood sample isolations 18 known pathogenic species from 10 fungal genera

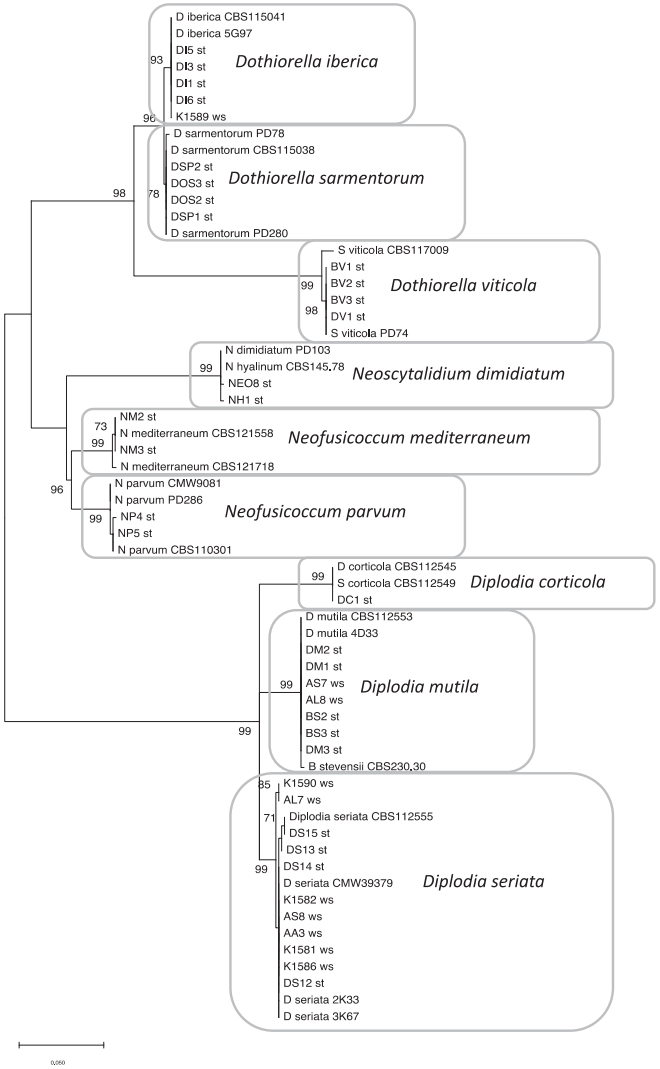

Fig. 1. Phylogenetic tree reconstructed by maximum-likelihood analysis from the concatenated sequences of the nuclear ribosomal internal transcribed spacer and translation elongation factor for 38 Botryosphaeriaceae taxa isolated from three almond orchards and one walnut orchard in Merced County, California, 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). st, spore trap; ws, wood sample.

(*Ceratocystis*, *Collophorina*, *Cytospora*, *Diaporthe*, *Diplodia*, *Dothiorella*, *Eutypa*, *Neofusicoccum*, *Neoscytalidium*, and *Pleurostoma*), plus two unidentified *Cytospora* and *Diaporthe* species. Comparison of the two sampling methods showed disparity in pathogen profiles (Table 3) with eight species found only in spore traps (*Diaporthe ampelina*, *Diaporthe eres*, *Diplodia corticola*, *Dothiorella sarmentorum*, *Dothiorella viticola*, *Neofusicoccum mediterraneum*, *Neofusicoccum parvum*, and *Neoscytalidium dimidiatum*), six found only in wood samples (*Ceratocystis destructans*, *Collophorina hispanica*, *Cytospora eucalypti*, *Diaporthe novem*, *Diaporthe rhusicola/chamaeropis*, and *P. richardsiae*), and four species (*Diplodia mutila*, *Diplodia seriata*, *Dothiorella Iberica*, and *E. lata*) found in both, although not consistently for all four orchards.

Discussion

Spore traps are commonly deployed to monitor airborne and rain-splashed spores. They have been used in the field of plant pathology to predict the risks of endemic or emerging crop pathogens, and prevent the incursion of regulated diseases in plant biosecurity (Jackson and Bayliss 2011). Passive spore-trapping methods, such as glass slides coated with petroleum jelly, have been largely used in perennial cropping systems to improve management guidelines for wood canker diseases, including timing of pruning and fungicide application (Eskalen et al. 2013; Molnar et al. 2020; Trouillas et al. 2012; Úrbez-Torres et al. 2010). These trapping methods 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 samplers) to study the epidemiology of wood canker diseases (Billones-Baaijens et al. 2018; Luo et al. 2020; van Niekerk et al. 2010). Active spore-trapping devices are more efficient at capturing bioaerosols and integrating fine-scale weather data 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 and 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; Úrbez-Torres et al. 2010; van Niekerk et al. 2010). During the course of our study, we also captured spores of Botryosphaeriaceae and Diaporthaceae after rain events. However, our focus was not to quantitatively record released spore inoculum and reinvestigate the cause and effect between precipitation events and amounts with pathogen spore counts in orchards. Instead, our goal was to test the hypothesis that the characteristics of the orchard impacted qualitative fungal inoculum and wood pathogen profiles.

The passive spore trapping deployed in orchards was a good predictor of the causal agents of wood canker diseases for some of the most widespread taxa at the genus level (*Diaporthe*, *Dothiorella*, and *Diplodia*) but overall failed to provide an accurate diagnosis at the species level. Only four species (*Dothiorella iberica*, *Diplodia seriata*, *Diplodia mutila*, and *E. lata*) were recovered from both spore traps and plant samples, albeit not consistently across the four orchards, whereas the remaining 14 species identified were unique to

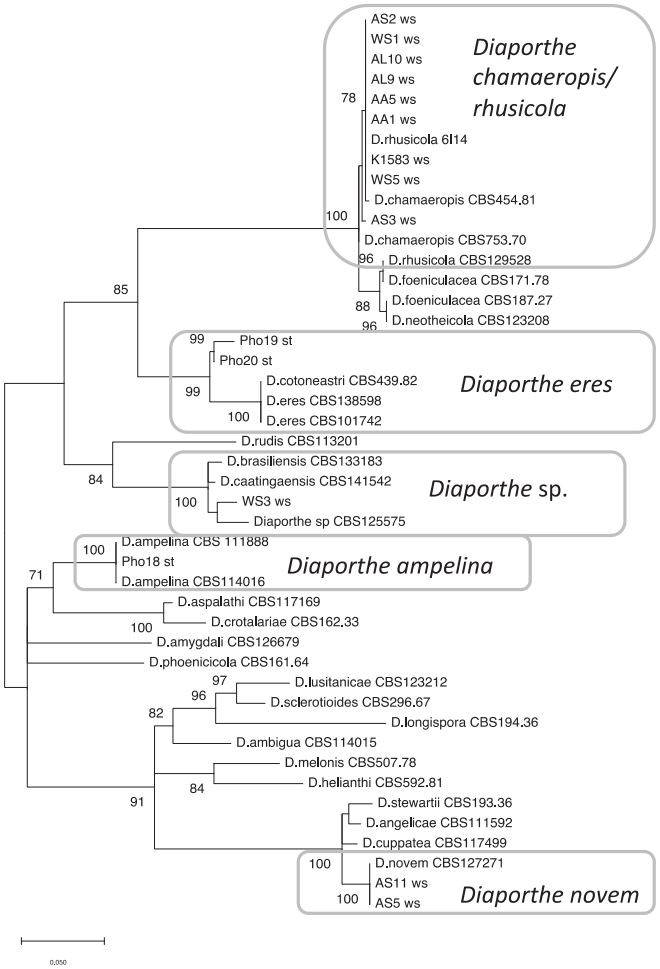

Fig. 2. Phylogenetic tree reconstructed by maximum-likelihood analysis from the concatenated sequences of the nuclear ribosomal internal transcribed spacer and translation elongation factor for 15 Diaporthaceae taxa recovered from three almond orchards and one walnut orchard in Merced County, California, and 30 *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). st, spore trap; ws, wood sample.

either diagnostic approach. The disparity between the two sampling methods cautions against using only spore-trapping data to predict the risks of wood canker diseases in nut crop orchards. One limit of our spore-trapping approach is that it only captures airborne and water-splashed fungi but overlooks pathogens vectored by insects. In line with previous reports (Holland et al. 2021), our diagnosis showed that most of the trunk and scaffold tree samples collected from almond orchards were infected with *Ceratocystis destructans*, which is reported to be predominantly vectored by insects (Moller and DeVay 1968). In addition, our sampling design was not suited to capture fungi with spatially restricted dispersal ability. Pycnidiospores of *Diaporthe* travel short distances after rain because they are exuded in a cirrus and are splash-dispersed (Cucuzza and Sall 1982; Linders et al. 1996). Thus, some species trapped in our sampling (i.e., *Diaporthe eres* and *Diaporthe ampelina*) may only represent localized infection of limb/scaffold (where glass slides were placed) of a single tree rather than species abundant in tree canopy (i.e., *Diaporthe chamaeropis/rhusicola*). Increasing the number of spore traps per orchard and/or placing them at various heights in the tree canopy might have resulted in a different outcome. Moreover, the strategy we adopted to select for a "representative" morphotype taxon likely excluded species because of the paucity of informative morphological characters (Gomes et al. 2013; Lawrence et al. 2015). This spore-trapping approach also skewed the results toward quantitatively abundant fungi that can quickly colonize culture medium. Hence, fungal groups such as *Pleurostoma* or *Collophorina* are less likely to be recovered in culture given their slow-growing nature and lower prevalence in orchards (Holland et al. 2021; Sohrabi et al. 2020), as supported by our plant sample diagnosis. Molecular methods (e.g., multiplex quantitative PCR, quantitative PCR using multispecies primers, nested PCR) have 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 robust experimental design for environmental samples with high-throughput sequencing technologies using customized fungal metabarcoding, will enable quick, sensitive, and reliable profiling for a range of fungal species and identify those capable of causing disease (Aguayo et al. 2018; Castaño et al. 2017; Morales-Cruz et al. 2018).

Despite the limitations of our method, the broad diversity of the fungal pathogens identified coincides with previous reports (Luo et al. 2020; Úrbez-Torres et al. 2010; van Niekerk et al. 2010). Our data suggested that airborne and water-splashed pathogenic inoculum in orchards was made up of endemic taxa from within the orchard as well as immigrant taxa originating from nearby riparian and cultivated areas. Immigrant taxa were characterized by rare trapping events (e.g., *Neofusicoccum*, *Neoscytalidium*). We speculate that those immigrant taxa came from cultivated and wild tree hosts at the perimeter of the orchard because Botryosphaeriaceae spores have been reported to travel only short distances (Ahimera et al. 2004; Michailides and Morgan 1993; Úrbez-Torres et al. 2010). In contrast, endemic taxa (e.g., *Dothiorella* in almond or *Diaporthe* in walnut) were characterized by frequent and repeated trapping events from orchards coupled with common isolation from symptomatic wood samples. Our data support the hypothesis that the incidence and frequency of endemic taxa was influenced by orchard type, age, and precipitation. For example, *Dothiorella* species appeared to be more specific to almond orchards and were increasingly trapped and reisolated as orchards aged. *Dothiorella sarmentorum* and *Dothiorella viticola* have been found in almond in California and Iran (Holland et al. 2021; Inderbitzin et al. 2010; Sohrabi et al. 2020). *Dothiorella iberica* has also been reported on almond in California (Doll et al. 2015; Holland et al. 2021), 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. 2014a, b; Eskalen et al. 2013; Úrbez-Torres and Gubler 2009; Úrbez-Torres et al. 2013) and likely originated initially from the surrounding vineyards and orchards. In addition, the incidence of *Dothiorella* was modulated by total precipitation and number of wet events as it was more frequently trapped in wet versus dry years in the same orchards (Almond orchards #1 and #2). These results are consistent with previous reports indicating a higher pathogen pressure after intense wet periods (Eskalen et al. 2013; Úrbez-Torres et al. 2010). Further, these findings suggest an important role of extreme wet conditions and changing weather patterns (e.g., El Niño) in disease outbreaks (Moral et al. 2019b).

In contrast, walnut appeared to be a preferred tree host to *Diaporthe* as spores were frequently and repeatedly trapped in both years of the trial, yet they were rarely trapped in the neighboring or

Table 2. Pathogen profile and incidence (in percent) from plant samples (n = 61) collected from the main frame of the tree (trunk/scaffold) or the canopy of the tree (branch/twig) within each orchard

Orchard type	Number of samples collected and tissue types	Fungal species isolated from plant samples[a]	Incidence in plant samples (%)[b]	Host tissue infected
Almond #1	Total = 16	*Ceratocystis destructans*	31	Trunk/scaffold
	Trunk/scaffold = 6	*Diaporthe rhusicola/chamaeropis*	31	Branch/twig
	Branch/twig = 10	*Diplodia seriata*	19	Branch/twig
		Cytospora eucalypti	6	Trunk/scaffold
		Dothiorella iberica	6	Trunk/scaffold
		Eutypa lata	6	Branch/twig
		Collophorina hispanica	6	Branch/twig
Almond #2	Total = 16	*Ceratocystis destructans*	19	Trunk/scaffold
	Trunk/scaffold = 6	*Diplodia seriata*	19	Trunk/scaffold – branch/twig
	Branch/twig = 10	*Diplodia mutila*	19	Trunk/scaffold – branch/twig
		Eutypa lata	19	Branch/twig
		Diaporthe rhusicola/chamaeropis	13	Branch/twig
		Cytospora eucalypti	6	Trunk/scaffold
		Dothiorella iberica	6	Trunk/scaffold
		Pleurostoma richardsiae	6	Branch/twig
Almond #3	Total = 16	*Diaporthe rhusicola/chamaeropis*	31	Branch/twig
	Trunk/scaffold = 5	*Diplodia seriata*	19	Trunk/scaffold – branch/twig
	Branch/twig = 11	*Ceratocystis destructans*	19	Trunk/scaffold
		Diplodia mutila	13	Branch/twig
		Diaporthe novem	13	Branch/twig
Walnut	Total = 13	*Diaporthe rhusicola/chamaeropis*	92	Branch/twig
	Branch/twig = 13	*Diaporthe* sp.	8	Branch/twig

[a] Fungi were identified to the species level based on phylogenetic analyses of internal transcribed spacer and translation elongation factor sequences. See Figures 1 and 2.
[b] Percent of samples testing positive for each pathogen (number of samples where fungal species were isolated/total number of samples × 100).

Table 3. Pathogen profile comparison between samples obtained from trees and spore traps

Orchard type	Fungal genus and species identified[a]	Taxon isolated from wood samples[b]	Taxon cultured from spore traps[b]
Almond #1	*Ceratocystis destructans*	Yes	–
	Collophorina hispanica	Yes	–
	Cytospora eucalypti	Yes	–
	Diaporthe ampelina	–	Yes
	Diaporthe rhusicola/chamaeropis	Yes	–
	Diplodia corticola	–	Yes
	Diplodia seriata	Yes	Yes
	Dothiorella iberica	Yes	Yes
	Dothiorella sarmentorum	–	Yes
	Eutypa lata	Yes	–
	Neoscytalidium dimidiatum	–	Yes
Almond #2	*Ceratocystis destructans*	Yes	–
	Cytospora eucalypti	Yes	–
	Cytospora sp.	–	Yes
	Diaporthe rhusicola/chamaeropis	Yes	–
	Diplodia mutila	Yes	Yes
	Diplodia seriata	Yes	Yes
	Dothiorella iberica	Yes	Yes
	Dothiorella sarmentorum	–	Yes
	Dothiorella viticola	–	Yes
	Eutypa lata	Yes	Yes
	Pleurostoma richardsiae	Yes	–
Almond #3	*Ceratocystis destructans*	Yes	–
	Diaporthe eres	–	Yes
	Diaporthe novem	Yes	–
	Diaporthe rhusicola/chamaeropis	Yes	–
Walnut	*Diaporthe ampelina*	–	Yes
	Diaporthe eres	–	Yes
	Diaporthe rhusicola/chamaeropis	Yes	–
	Diaporthe sp.	Yes	–
	Diplodia mutila	–	Yes
	Diplodia seriata	–	Yes
	Neofusicoccum mediterraneum	–	Yes
	Neofusicoccum parvum	–	Yes
	Neoscytalidium dimidiatum	–	Yes

[a] Fungi were identified to the species level based on phylogenetic analyses of internal transcribed spacer and translation elongation factor sequences. See Figures 1 and 2.
[b] "–" indicates taxon was not found.

older almond orchards. *Diaporthe chamaeropis/rhusicola* was the dominant taxon identified in plant samples, but species identification could not be resolved with the two loci sequenced. To our knowledge, *Diaporthe chamaeropis* has never been identified as a pathogen of walnut but has been associated with almond, pistachio, and grapevine in California (Chen et al. 2014b; Holland et al. 2021; Lawrence et al. 2015), making it a plausible candidate. The closely related *Diaporthe rhusicola* and *Diaporthe neotheicola* have commonly been reported on walnut along with several other *Diaporthe* species (Chen et al. 2014a; Fan et al. 2018; Fang et al. 2020; Jiménez Luna et al. 2020; López-Moral et al. 2020). However, surveys from walnut orchards in both California and Spain (Chen et al. 2014a; López-Moral et al. 2020) showed that the Botryosphaeriaceae were the most frequently isolated group, which differed from our results, likely because of our small orchard sampling size. In contrast with *Diaporthe* and *Dothiorella*, *Diplodia seriata* and *Diplodia mutila* were not host-specific and were trapped in both walnut and almond orchards and isolated from all almond tree parts. These results are not surprising given the known broad host range of these species, and that they have been commonly isolated from trees in all major almond- and walnut-producing countries (Chen et al. 2014a; Holland et al. 2021; López-Moral et al. 2020; Olmo et al. 2016; Sohrabi et al. 2020). Spore trapping coupled with wood sampling will need to be extended to additional almond and walnut orchards grown in different locations in California, with contrasting climates to support the findings and hypotheses outlined in this work.

Our study highlights the limits of our spore-trapping strategy and diagnosis method to assess the disease risks caused by wood canker pathogens in almond and walnut orchards. Passive spore traps offer a cost-effective method to profile the dominant airborne and water-splashed wood canker pathogens at the genus level and provide supporting evidence that under dry conditions, infection risks are minimal. However, this information should be complemented with fungal profiles from insect traps to capture the risks posed by wood canker pathogens vectored by insects. In addition, increasing the number of spore traps per orchard and location in trees coupled with high-throughput sequencing capabilities will refine the disease risk predictions. Our data support early adoption of management practices that include both a fungicide and an insecticide program to reduce the likelihood of pathogen infection in young trees, thereby increasing the long-term productivity and profitability as shown for vineyards (Gispert et al. 2020).

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