Identification and Characterization of *Neofabraea kienholzii* and *Phlyctema vagabunda* Causing Leaf and Shoot Lesions of Olive in California

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Abstract

California produces over 95% of the olives grown in the United States. In 2017, California's total bearing acreage for olives was 14,570 hectares producing 192,000 tons of olives valued at \$186.6 million. During the early spring of 2016, unusual leaf and shoot lesions were detected in olive trees from superhigh-density orchards in the Northern San Joaquin and Sacramento valleys of California. Affected trees displayed numerous leaf and shoot lesions developing at wounds created by mechanical harvesters. The 'Arbosana' cultivar was highly affected by the disease, whereas the disease was sporadic in 'Arbequina' and not found in 'Koroneiki' cultivar.

Two fungal species, *Neofabraea kienholzii* and *Phlyctema vagabunda*, were found to be consistently associated with the disease, and Koch's postulates were completed. Species identity was confirmed by morphology and molecular data of the partial large subunit rDNA, the internal transcribed spacer region, and partial beta-tubulin region. The disease signs and symptoms are described and illustrated.

Keywords: olive, super-high-density, fungi, phylogeny, *Phlyctema*, *Neofabraea*, lesion

The olive tree, *Olea europaea* subsp. *europaea* L., is native to the Mediterranean basin and is one of the oldest domesticated and most extensively cultivated plant species in the world. This species has been a part of Mediterranean civilizations for thousands of years and is utilized for cultural, medical, and nutritional purposes (Connell 2005). Olive trees thrive in a Mediterranean climate. Spain, Italy, Greece, Turkey, Tunisia, and Morocco are major producers, but olives are also cultivated outside the Mediterranean region in areas with an appropriate climate: a long, hot growing season and a relatively cool winter (Connell 2005). Hundreds of cultivars have been selected within the Mediterranean countries, with some being best adapted for oil production, others for table uses, and others being suitable for either use depending on the relative prices of oil and table olives (Vossen 2007).

The increasing interest for olive oil beyond the Mediterranean regions and recent emphasis on the benefits of olive oil to human health have led to rising consumer demand, with global consumption and production of olive oil almost doubling over the last 25 years. The U.S. consumption of olive oil has tripled over the past two decades, making it the third largest consumer of olive oil worldwide. Despite this increase in consumption, the U.S. production of olive oil has remained low. California produces more than 95% of the olives grown in the nation, accounting for 5.8% of the olive oil consumed

in the United States. Olive production also occurs in Arizona, Florida, Georgia, and Texas (<https://www.nass.usda.gov/>).

In 2017, the total bearing acreage for table and oil olives in California was 14,570 hectares with a total production of 192,000 tons valued at \$186.6 million (<https://www.nass.usda.gov/>). California's flourishing olive oil industry is illustrated by high demand and acreage that continues to grow as new, higher-yielding orchards are being planted statewide. With the escalating cost of labor, olive growers in California are relying more on mechanized practices to deliver enough olive supply while remaining profitable (Nguyen et al. 2017; Tous et al. 2010; Vossen 2007). High-density (HD) plantings and superhigh-density (SHD) olive orchards have become common practices in California. SHD production systems are using canopy-contact harvesters adapted from grape harvesters (Tous et al. 2010; Vossen 2007), thus producing more wounds for pathogen infection and ultimately increasing the risks for disease occurrence, spread, and emergence (Moral et al. 2012).

The main aerial diseases of olive include olive knot caused by *Pseudomonas savastanoi* pv. *savastanoi*, peacock spot (synonyms: bird's eye spot and olive leaf spot) caused by *Venturia oleaginea* (Castagne) Rossman & Crous (synonyms: *Fusicladium oleagineum* (Castagne) Ritschel & U. Braun and *Spilocaea oleaginea* (Castagne) S. Hughes), Cercospora leaf spot caused by *Pseudocercospora cladosporioides* (Sacc.) U. Braun (synonyms: *Myco-centrospora cladosporioides* (Sacc.) Deighton and *Cercospora cladosporioides* Sacc.), and olive anthracnose caused by several species of *Colletotrichum* (Moral et al. 2014). Recently, several ascomycetous fungi belonging to the *Botryosphaeriaceae*, *Cytosporaceae*, *Diaporthaceae*, *Diatrypaceae*, *Togniniaceae*, and a few basidiomycetous fungi (*Schizophyllum commune* Fr. and *Trametes versicolor* (L.) Lloyd) were found associated with twig and branch dieback of olive in California (Lawrence et al. 2018; Urbez-Torres et al. 2013).

While oil olives are a growing industry in California, olive production could be challenged by emerging new diseases as more intensive, higher-yielding, and mechanized production practices are being adopted statewide. In Spain and Portugal, the fungi *Phlyctema*

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vagabunda Desm. (syn: *Neofabraea alba* (E.J. Guthrie) Verkley and *Neofabraea vagabunda* (Desm.) Rossman) were shown recently to cause branch cankers and twig dieback in ‘Arbequina’ and ‘Picual’ cultivars as well as small, circular, and necrotic lesions on leaves and fruit rot (Romero et al. 2015, 2016, 2018). Romero et al. (2018) argued that the recent increase of symptoms caused by *P. vagabunda* in these Mediterranean regions could be related to the intensification and expansion of olive production, which has led to increased wounding of trees due to mechanical harvesting and pruning in autumn and early winter, respectively. In California, *P. vagabunda* was reported to cause fruit spot on ‘Coratina’ and ‘Picholine’ cultivars in two commercial olive orchards in Sonoma County (Rooney-Latham et al. 2013). Olive fruit rot caused by *P. vagabunda* is well known in Italy and Spain as olive leprosy (Petri 1915; Roca et al. 2007).

P. vagabunda has been assigned previously to the genera *Pezizula* Tul. & C. Tul., *Neofabraea*, or *Phlyctema* (Chen et al. 2016; Johnston et al. 2014; Verkley 1999). Historically, it was known commonly as *N. alba* (anamorph *Phlyctema vagabunda*) or *Gloeosporium album* Osterw., however, a recent taxonomic study proposed its name to be *N. vagabunda*, maintaining *N. alba* as a synonym (Johnston et al. 2014). Later, Chen et al. (2016) suggested that *Phlyctema* and *Neofabraea* species represented distinct lineages based on phylogenetic placement and distinct morphologies. The causal agent of olive leprosy has been reclassified several times, first as *P. vagabunda* (von Arx 1970), then as *N. alba* (Verkley 1999), and finally as *N. vagabunda* (Johnston et al. 2014). Currently, the accepted name for this species is *P. vagabunda*, the original name (Desmazières 1847).

During the early spring of 2016, necrotic leaf lesions, lesions in shoots, and occasionally cankers in branches were observed in two SHD oil olive orchards in San Joaquin County in California. Shoot lesions as well as branch cankers occurred at wounds caused by mechanical harvesters. The cultivar ‘Arbosana’ was highly affected by the disease, eventually suffering severe defoliation. Infected olive tissues were taken to the laboratory for isolations of putative microorganisms and disease diagnosis. Results of isolations revealed two *Neofabraea*-like species that were consistently isolated from the different symptomatic tissues. To date, little is known about the diversity and biology of *Neofabraea* and *Phlyctema* pathogens affecting olives in California as well as the symptoms, damage, and yield loss these fungi may cause in olive trees. Also, the discovery of a new, apparently emerging disease in California olive orchards is of major concern, as it potentially constitutes a new threat to the developing olive oil industry. The present study aimed to investigate various aspects of this emerging disease including disease distribution, etiology and epidemiology, pathogen biology, and host susceptibility.

Materials and Methods

Surveys and isolation of fungal strains. Surveys of table and oil olive orchards were conducted in Colusa, Contra Costa, Glenn, Kings, San Joaquin, Stanislaus, and Tulare counties. Orchards surveyed included traditional as well as HD and SHD plantations, totaling 27 orchards. Approximately 30 to 40 olive trees per orchard were inspected for the presence of leaf and shoot lesions typical of the new disease. Symptomatic olive tissues were taken to the laboratory to proceed with microbiological isolations to determine which pathogens may be present. Isolations were made using small pieces (3 × 3 mm) of leaves and shoots taken from the margin between necrotic and apparently healthy tissues. Plant pieces were submerged in 0.5% sodium hypochlorite for 2 min, rinsed twice in sterile water, and plated onto acidified potato dextrose agar (APDA; 2.6 ml of 25% [vol/vol] lactic acid per liter of medium) filled petri plates for isolation of fungi. Plates were incubated in the laboratory at room temperature (around 24°C) under an approximate 12-h photoperiod, and fungal growth was observed after 7 and 14 days. Fungal colonies consistently emerging from plated tissue pieces were transferred to potato dextrose agar (PDA) plates. Hyphal tips were then selected and plated onto new PDA plates in order to obtain pure cultures for use in taxonomic and biological studies. Isolates collected for this study are reported in Table 1, and all are maintained in the culture

collection of the Department of Plant Pathology of the University of California, Davis at the Kearney Agricultural Research and Extension Center (KARE), Parlier, CA, USA.

DNA extraction, PCR amplification, sequencing, and phylogenetic analyses. Total genomic DNA was extracted from 14-day-old colonies on PDA by scraping the mycelium with a sterile scalpel and following the FastDNA Spin Kit manufacturer’s protocol (MP Biomedicals, Burlingame, CA, USA). The primer pairs ITS1/ITS4 (White et al. 1990) and Bt-T2m-Up and Bt-LEV-Lo1 (de Jong et al. 2001) were used to amplify the nuclear rDNA internal transcribed spacer (ITS) region including ITS1, the intervening 5.8S rRNA gene, and ITS2, and portions of the beta-tubulin (*TUB2*), respectively. A segment of the large subunit ribosomal DNA (LSU) gene was amplified with primers LR0R and LR5 (Vilgalys and Hester 1990). PCR reactions were conducted using the conditions described by Zhu et al. (2012). Amplification products were visually checked on a 1.5% agarose gel for presence and size via electrophoresis (120 V for 25 min) and UV illumination followed by purification using ExoSap-IT Product Cleanup Reagent (Affymetrix, Santa Clara, CA, USA). PCR amplicons were sequenced bidirectionally using an ABI 3730 Capillary Electrophoresis Genetic Analyzer (College of Biological Sciences Sequencing Facility, University of California, Davis).

Forward and reverse nucleotide sequences were assembled, proofread, and edited in Sequencher v. 5 (Gene Codes Corporation, Ann Arbor, MI, USA) and deposited in GenBank (Table 1). Homologous sequences with high similarity from ex-type and nontype fungal specimens in the *Dematiaceae* (*Helotiales*, *Leotiomyces*) were included for phylogenetic reference utilizing the BLASTn function in NCBI and literature review. Multiple sequence alignments were performed in MEGA v. 6 (Tamura et al. 2013) and manually adjusted where necessary in Mesquite v. 3.10 (Maddison and Maddison 2016). Alignments were submitted to TreeBASE under accession number S23539. Maximum parsimony (MP) and maximum likelihood (ML) analyses were performed on individual gene datasets and the three-gene (ITS+LSU+*TUB2*) combined dataset in PAUP 4.0b162 and MEGA 6, respectively (Swofford 2002; Tamura et al. 2013). For MP analyses, heuristic searches with 1,000 random sequence additions were implemented with the Tree-Bisection-Reconnection algorithm; gaps were treated as missing data. Bootstrap analyses with 1,000 replicates were used to estimate branch support. For ML analyses, MEGA 6 was used to infer a model of nucleotide evolution for each dataset, using the Akaike Information Criterion (AIC). ML analyses utilized the Nearest-Neighbor-Interchange heuristic method, and branch support was determined by 1,000 bootstrap replicates. Sequences of *Infundichalara microchona* (W. Gams) Réblová & W. Gams isolate CBS 175.74 (*Helotiales*, *Incertae sedis*) served as the outgroup taxon in the phylogenetic analyses.

Morphological characterization. The asexual morphs of the newly reported species were examined and described. Fungal isolates (*N. kienholzii* isolates KARE943 and KARE1291; *P. vagabunda* isolates KARE1943 and KARE1944) were cultured on PDA and tomato agar (TA) (Cameldi et al. 2017) petri plates incubated at the laboratory’s ambient conditions (24°C). Culture morphology, culture color (Rayner 1970) and colony growth rate, type of fruiting body as well as size and shape of conidia were recorded. Colony color and morphology was examined after 7 and 24 days. Fungal specimens were mounted in sterile water on a glass slide and examined with a Leica compound microscope (Leica DM2000 LED Microscope, Wetzlar, Germany). Measurements of 30 conidia including length and width for each of three isolates per fungal species were obtained at 400× magnification. Fungal fruiting bodies occurring naturally on olive leaves ‘Arbosana’ in the field were also examined for microscopic characteristics using a Leica M80 stereomicroscope.

The optimum temperature for growth of three representative strains of each fungal species was determined as described by Cameldi et al. (2017). A 5-mm mycelial plug taken from the margin of an actively growing colony was placed in the center of an 85-mm diameter TA petri plate. Cultures of four replicates for each isolate were

incubated at temperatures ranging from 5 to 30°C in five-degree increments. Plates were incubated in the dark, and two measurements of colony diameter at right angles to each other were taken weekly for 3 weeks. Average colony diameters per 21 days of growth were obtained at each of the six temperatures tested (5, 10, 15, 20, 25, and 30°C). The experiment was conducted twice.

Pathogenicity tests. *Leaf pathogenicity assays.* Pathogenicity studies were conducted in November 2016 and November 2017 to determine the ability of the newly reported fungi to cause lesions

in olive leaves. Two- to three-year-old potted olive saplings of the ‘Arbequina’, ‘Arbosana’, and ‘Koroneiki’ cultivars were used for these assays, and plants were maintained in a lath house at the University of California, Davis.

In the 2016 experiment, leaves were inoculated following wounding with a sterile needle (via puncturing) as well as without wounding to evaluate the necessity of wounds for infection. Inocula of *N. kienholzii* (isolates KARE1066 and KARE1068) and *P. vagabunda* (isolates KARE944 and KARE607) were produced by culturing OA

Table 1. *Neofabraea kienholzii* and *Phlyctema vagabunda* isolates collected and used in this study

Species	Isolate	Cultivar	Substrate	Location	GenBank accession numbers ^a		
					ITS	LSU	TUB2
<i>Neofabraea kienholzii</i>	KARE1006	Arbosana	Leaf	San Joaquin Co., CA, USA	MK174721	MK160188	MK174830
<i>N. kienholzii</i>	KARE1007	Arbosana	Leaf	San Joaquin Co., CA, USA	MK174722	MK160189	MK174831
<i>N. kienholzii</i>	KARE1009	Arbosana	Shoot	San Joaquin Co., CA, USA	MK174723	MK160190	MK174832
<i>N. kienholzii</i>	KARE1010	Arbosana	Shoot	San Joaquin Co., CA, USA	MK174724	MK160191	MK174833
<i>N. kienholzii</i>	KARE1011	Arbosana	Shoot	San Joaquin Co., CA, USA	MK174725	MK160192	MK174834
<i>N. kienholzii</i>	KARE1012	Arbosana	Shoot	San Joaquin Co., CA, USA	MK174726	MK160193	MK174835
<i>N. kienholzii</i>	KARE1014	Arbosana	Shoot	San Joaquin Co., CA, USA	MK174727	MK160194	MK174836
<i>N. kienholzii</i>	KARE1015	Arbosana	Shoot	San Joaquin Co., CA, USA	MK174728	MK160195	MK174837
<i>N. kienholzii</i>	KARE1016	Arbosana	Shoot	San Joaquin Co., CA, USA	MK174729	MK160196	MK174838
<i>N. kienholzii</i>	KARE1017	Arbosana	Shoot	San Joaquin Co., CA, USA	MK174730	MK160197	MK174839
<i>N. kienholzii</i>	KARE1018	Arbosana	Shoot	San Joaquin Co., CA, USA	MK174731	MK160198	MK174840
<i>N. kienholzii</i>	KARE1019	Arbosana	Shoot	San Joaquin Co., CA, USA	MK174732	MK160199	MK174841
<i>N. kienholzii</i>	KARE1022	Arbosana	Leaf	San Joaquin Co., CA, USA	MK174733	MK160200	MK174842
<i>N. kienholzii</i>	KARE1023	Arbosana	Leaf	San Joaquin Co., CA, USA	MK174734	MK160201	MK174843
<i>N. kienholzii</i>	KARE1024	Arbosana	Leaf	San Joaquin Co., CA, USA	MK174735	MK160202	MK174844
<i>N. kienholzii</i>	KARE1025	Arbosana	Shoot	San Joaquin Co., CA, USA	MK174736	MK160203	MK174845
<i>N. kienholzii</i>	KARE1028	Arbosana	Shoot	San Joaquin Co., CA, USA	MK174737	MK160204	MK174846
<i>N. kienholzii</i>	KARE1029	Arbosana	Shoot	San Joaquin Co., CA, USA	MK174738	MK160205	MK174847
<i>N. kienholzii</i>	KARE1030	Arbosana	Shoot	San Joaquin Co., CA, USA	MK174739	MK160206	MK174848
<i>N. kienholzii</i>	KARE1031	Arbosana	Shoot	San Joaquin Co., CA, USA	MK174740	MK160207	MK174849
<i>N. kienholzii</i>	KARE1032	Arbosana	Shoot	San Joaquin Co., CA, USA	MK174741	MK160208	MK174850
<i>N. kienholzii</i>	KARE1033	Arbosana	Shoot	San Joaquin Co., CA, USA	MK174742	MK160209	MK174851
<i>N. kienholzii</i>	KARE1065	Arbosana	Leaf	San Joaquin Co., CA, USA	MK174743	MK160210	MK174852
<i>N. kienholzii</i>	KARE1066 ^b	Arbosana	Leaf	San Joaquin Co., CA, USA	MK174744	MK160211	MK174853
<i>N. kienholzii</i>	KARE1067	Arbosana	Leaf	San Joaquin Co., CA, USA	MK174745	MK160212	MK174854
<i>N. kienholzii</i>	KARE1068 ^b	Arbosana	Leaf	San Joaquin Co., CA, USA	MK174746	MK160213	MK174855
<i>N. kienholzii</i>	KARE1069	Arbosana	Leaf	San Joaquin Co., CA, USA	MK174747	MK160214	MK174856
<i>N. kienholzii</i>	KARE1070	Arbosana	Leaf	San Joaquin Co., CA, USA	MK174748	MK160215	MK174857
<i>N. kienholzii</i>	KARE1071	Arbosana	Leaf	San Joaquin Co., CA, USA	MK174749	MK160216	MK174858
<i>N. kienholzii</i>	KARE1072	Arbosana	Leaf	San Joaquin Co., CA, USA	MK174750	MK160217	MK174859
<i>N. kienholzii</i>	KARE1073	Arbosana	Leaf	San Joaquin Co., CA, USA	MK174751	MK160218	MK174860
<i>N. kienholzii</i>	KARE1074	Arbosana	Leaf	San Joaquin Co., CA, USA	MK174752	MK160219	MK174861
<i>N. kienholzii</i>	KARE1075	Arbosana	Leaf	San Joaquin Co., CA, USA	MK174753	MK160220	MK174862
<i>N. kienholzii</i>	KARE1076	Arbosana	Leaf	San Joaquin Co., CA, USA	MK174754	MK160221	MK174863
<i>N. kienholzii</i>	KARE1077	Arbosana	Leaf	San Joaquin Co., CA, USA	MK174755	MK160222	MK174864
<i>N. kienholzii</i>	KARE1078	Arbosana	Leaf	San Joaquin Co., CA, USA	MK174756	MK160223	MK174865
<i>N. kienholzii</i>	KARE1079	Arbosana	Leaf	San Joaquin Co., CA, USA	MK174757	MK160224	MK174866
<i>N. kienholzii</i>	KARE1080	Arbosana	Leaf	San Joaquin Co., CA, USA	MK174758	MK160225	MK174867
<i>N. kienholzii</i>	KARE1082	Arbosana	Leaf	San Joaquin Co., CA, USA	MK174759	MK160226	MK174868
<i>N. kienholzii</i>	KARE1083	Arbosana	Leaf	San Joaquin Co., CA, USA	MK174760	MK160227	MK174869
<i>N. kienholzii</i>	KARE1084	Arbosana	Leaf	San Joaquin Co., CA, USA	MK174761	MK160228	MK174870
<i>N. kienholzii</i>	KARE1085	Arbosana	Leaf	San Joaquin Co., CA, USA	MK174762	MK160229	MK174871
<i>N. kienholzii</i>	KARE1281 ^b	Arbosana	Leaf	San Joaquin Co., CA, USA	MK174763	MK160230	MK174872
<i>N. kienholzii</i>	KARE1282	Arbosana	Leaf	San Joaquin Co., CA, USA	MK174764	MK160231	MK174873
<i>N. kienholzii</i>	KARE1292	Arbosana	Shoot	Glenn Co., CA, USA	MK174765	MK160232	MK174874
<i>N. kienholzii</i>	KARE1556	Arbosana	Shoot	San Joaquin Co., CA, USA	MK174766	MK160233	MK174875
<i>N. kienholzii</i>	KARE1557	Arbosana	Shoot	San Joaquin Co., CA, USA	MK174767	MK160234	MK174876
<i>N. kienholzii</i>	KARE1558	Arbosana	Shoot	San Joaquin Co., CA, USA	MK174768	MK160235	MK174877
<i>N. kienholzii</i>	KARE1561	Arbosana	Shoot	San Joaquin Co., CA, USA	MK174769	MK160236	MK174878
<i>N. kienholzii</i>	KARE1562	Arbosana	Shoot	San Joaquin Co., CA, USA	MK174770	MK160237	MK174879
<i>N. kienholzii</i>	KARE1563	Arbosana	Shoot	San Joaquin Co., CA, USA	MK174771	MK160238	MK174880
<i>N. kienholzii</i>	KARE1564	Arbosana	Shoot	San Joaquin Co., CA, USA	MK174772	MK160239	MK174881

(Continued on next page)

^a ITS = internal transcribed spacer, LSU = partial large subunit rDNA, TUB2 = β -tubulin.

^b Isolates used for pathogenicity tests in this study.

petri plates with the various isolates and incubating them for 14 days at 20°C in the dark. *N. kienholzii* isolates produced conidia profusely in OA medium. Accordingly, conidial inoculum was used for inoculation of *N. kienholzii* isolates. In contrast, *P. vagabunda* isolates did not produce conidia on the tested culture media (OA, PDA, and TA). For this reason, mycelial fragments were used to serve as inoculum for *P. vagabunda*. Mycelium was harvested from the surface of

15-day-old colonies using a sterile scalpel blade and then mixed in sterile water to be ground as well as homogenized using a common food blender. Both conidial and mycelial inocula of *N. kienholzii* and *P. vagabunda*, respectively, were suspended in sterile water, and the resulting suspension was adjusted to a final concentration of 1×10^5 propagules per ml. Twenty microliters of the inoculum suspension was used to inoculate freshly wounded and unwounded

Table 1. (Continued from previous page)

Species	Isolate	Cultivar	Substrate	Location	GenBank accession numbers ^a		
					ITS	LSU	TUB2
<i>N. kienholzii</i>	KARE1565	Arbosana	Shoot	San Joaquin Co., CA, USA	MK174773	MK160240	MK174882
<i>N. kienholzii</i>	KARE942	Arbosana	Shoot	San Joaquin Co., CA, USA	MK174774	MK160241	MK174883
<i>N. kienholzii</i>	KARE943 ^b	Arbosana	Shoot	San Joaquin Co., CA, USA	MK174775	MK160242	MK174884
<i>N. kienholzii</i>	KARE946	Arbosana	Shoot	San Joaquin Co., CA, USA	MK174776	MK160243	MK174885
<i>N. kienholzii</i>	KARE947	Arbosana	Shoot	San Joaquin Co., CA, USA	MK174777	MK160244	MK174886
<i>N. kienholzii</i>	KARE950	Arbosana	Shoot	San Joaquin Co., CA, USA	MK174778	MK160245	MK174887
<i>N. kienholzii</i>	KARE951	Arbosana	Shoot	San Joaquin Co., CA, USA	MK174779	MK160246	MK174888
<i>N. kienholzii</i>	KARE1649	Arbequina	Fruit	San Joaquin Co., CA, USA	MK174780	MK160247	MK174889
<i>N. kienholzii</i>	KARE1651	Arbequina	Fruit	San Joaquin Co., CA, USA	MK174781	MK160248	MK174890
<i>N. kienholzii</i>	KARE1946	Arbequina	Leaf	San Joaquin Co., CA, USA	MK174782	MK160249	MK174891
<i>N. kienholzii</i>	KARE1947	Arbequina	Leaf	San Joaquin Co., CA, USA	MK174783	MK160250	MK174892
<i>N. kienholzii</i>	KARE1948	Arbequina	Leaf	San Joaquin Co., CA, USA	MK174784	MK160251	MK174893
<i>N. kienholzii</i>	KARE1949	Arbequina	Leaf	San Joaquin Co., CA, USA	MK174785	MK160252	MK174894
<i>N. kienholzii</i>	KARE1950	Arbequina	Leaf	San Joaquin Co., CA, USA	MK174786	MK160253	MK174895
<i>N. kienholzii</i>	KARE1951	Arbequina	Leaf	San Joaquin Co., CA, USA	MK174787	MK160254	MK174896
<i>Phlyctema vagabunda</i>	KARE1642	Arbequina	Fruit	San Joaquin Co., CA, USA	MK174679	MK160146	MK174788
<i>P. vagabunda</i>	KARE1643	Arbequina	Fruit	San Joaquin Co., CA, USA	MK174680	MK160147	MK174789
<i>P. vagabunda</i>	KARE1644	Arbequina	Fruit	San Joaquin Co., CA, USA	MK174681	MK160148	MK174790
<i>P. vagabunda</i>	KARE1645	Arbequina	Fruit	San Joaquin Co., CA, USA	MK174682	MK160149	MK174791
<i>P. vagabunda</i>	KARE1646	Arbequina	Fruit	San Joaquin Co., CA, USA	MK174683	MK160150	MK174792
<i>P. vagabunda</i>	KARE1647	Arbequina	Fruit	San Joaquin Co., CA, USA	MK174684	MK160151	MK174793
<i>P. vagabunda</i>	KARE1648	Arbequina	Fruit	San Joaquin Co., CA, USA	MK174685	MK160152	MK174794
<i>P. vagabunda</i>	KARE1650	Arbequina	Fruit	San Joaquin Co., CA, USA	MK174686	MK160153	MK174795
<i>P. vagabunda</i>	KARE1652	Arbequina	Fruit	San Joaquin Co., CA, USA	MK174687	MK160154	MK174796
<i>P. vagabunda</i>	KARE1653	Arbequina	Fruit	San Joaquin Co., CA, USA	MK174688	MK160155	MK174797
<i>P. vagabunda</i>	KARE1654	Arbequina	Fruit	San Joaquin Co., CA, USA	MK174689	MK160156	MK174798
<i>P. vagabunda</i>	KARE1655	Arbequina	Fruit	San Joaquin Co., CA, USA	MK174690	MK160157	MK174799
<i>P. vagabunda</i>	KARE1656	Arbequina	Fruit	San Joaquin Co., CA, USA	MK174691	MK160158	MK174800
<i>P. vagabunda</i>	KARE1657	Arbequina	Fruit	San Joaquin Co., CA, USA	MK174692	MK160159	MK174801
<i>P. vagabunda</i>	KARE1658	Arbequina	Fruit	San Joaquin Co., CA, USA	MK174693	MK160160	MK174802
<i>P. vagabunda</i>	KARE1659	Arbequina	Fruit	San Joaquin Co., CA, USA	MK174694	MK160161	MK174803
<i>P. vagabunda</i>	KARE1660	Arbequina	Fruit	San Joaquin Co., CA, USA	MK174695	MK160162	MK174804
<i>P. vagabunda</i>	KARE1943 ^b	Arbequina	Fruit	San Joaquin Co., CA, USA	MK174696	MK160163	MK174805
<i>P. vagabunda</i>	KARE1944 ^b	Arbequina	Fruit	San Joaquin Co., CA, USA	MK174697	MK160164	MK174806
<i>P. vagabunda</i>	KARE1945	Arbequina	Fruit	San Joaquin Co., CA, USA	MK174698	MK160165	MK174807
<i>P. vagabunda</i>	KARE1952	Arbequina	Leaf	San Joaquin Co., CA, USA	MK174699	MK160166	MK174808
<i>P. vagabunda</i>	KARE1953	Arbequina	Leaf	San Joaquin Co., CA, USA	MK174700	MK160167	MK174809
<i>P. vagabunda</i>	KARE606	Arbosana	Leaf	San Joaquin Co., CA, USA	MK174701	MK160168	MK174810
<i>P. vagabunda</i>	KARE607 ^b	Arbosana	Shoot	San Joaquin Co., CA, USA	MK174702	MK160169	MK174811
<i>P. vagabunda</i>	KARE610	Arbosana	Leaf	San Joaquin Co., CA, USA	MK174703	MK160170	MK174812
<i>P. vagabunda</i>	KARE1013	Arbosana	Shoot	San Joaquin Co., CA, USA	MK174704	MK160171	MK174813
<i>P. vagabunda</i>	KARE1026	Arbosana	Shoot	San Joaquin Co., CA, USA	MK174705	MK160172	MK174814
<i>P. vagabunda</i>	KARE1081	Arbosana	Leaf	San Joaquin Co., CA, USA	MK174706	MK160173	MK174815
<i>P. vagabunda</i>	KARE1086	Arbosana	Leaf	San Joaquin Co., CA, USA	MK174707	MK160174	MK174816
<i>P. vagabunda</i>	KARE1287	Arbosana	Shoot	Glenn Co., CA, USA	MK174708	MK160175	MK174817
<i>P. vagabunda</i>	KARE1288	Arbosana	Shoot	Glenn Co., CA, USA	MK174709	MK160176	MK174818
<i>P. vagabunda</i>	KARE1289	Arbosana	Shoot	Glenn Co., CA, USA	MK174710	MK160177	MK174819
<i>P. vagabunda</i>	KARE1291	Arbosana	Shoot	Glenn Co., CA, USA	MK174711	MK160178	MK174820
<i>P. vagabunda</i>	KARE1293	Arbosana	Shoot	Glenn Co., CA, USA	MK174712	MK160179	MK174821
<i>P. vagabunda</i>	KARE1294	Arbosana	Shoot	Glenn Co., CA, USA	MK174713	MK160180	MK174822
<i>P. vagabunda</i>	KARE1295	Arbosana	Shoot	Glenn Co., CA, USA	MK174714	MK160181	MK174823
<i>P. vagabunda</i>	KARE611	Arbosana	Leaf	San Joaquin Co., CA, USA	MK174715	MK160182	MK174824
<i>P. vagabunda</i>	KARE944 ^b	Arbosana	Shoot	San Joaquin Co., CA, USA	MK174716	MK160183	MK174825
<i>P. vagabunda</i>	KARE945	Arbosana	Shoot	San Joaquin Co., CA, USA	MK174717	MK160184	MK174826
<i>P. vagabunda</i>	KARE948	Arbosana	Shoot	San Joaquin Co., CA, USA	MK174718	MK160185	MK174827
<i>P. vagabunda</i>	KARE949	Arbosana	Shoot	San Joaquin Co., CA, USA	MK174719	MK160186	MK174828
<i>P. vagabunda</i>	KARE955	Arbosana	Leaf	San Joaquin Co., CA, USA	MK174720	MK160187	MK174829

leaves using 10-leaf replicates, respectively. Because the two fungal species were inoculated using different source inocula (mycelium versus conidia), the effect of both species on pathogenicity was analyzed separately. Thus, for each species, the effect of isolate and cultivar on disease severity was subjected to analysis of variance (ANOVA). The treatment means were compared by Tukey's HSD test (honestly significant difference) at $P = 0.05$. In the case of *P. vagabunda* inoculations, inverse transformation of the lesion size was applied to satisfy the normality and homogeneity of the variance requirements of ANOVA. Data from all experiments were analyzed using Statistix 10 (Analytical Software, Tallahassee, FL, USA). The experiment was conducted twice using different isolates of each species.

In the 2017 experiment, conidial suspensions for both *N. kienholzii* and *P. vagabunda* were obtained and used for leaf inoculation. Conidia of *N. kienholzii* were produced as described above, whereas *P. vagabunda* conidia were obtained following artificial inoculation of 'Gordal Sevillana' olive fruits. Ripened olive fruits harvested at the Kearney Agricultural Research and Extension Center were surface disinfested by immersion in 0.5% sodium hypochlorite solution for 2 min, rinsed twice with sterile water, and blotted dry with a paper towel. Fruits were then wound-inoculated with a mycelial plug of PDA mounted on a sterile needle, and the plug was left on the surface of the fruit until the end of the incubation period. Inoculated fruits were incubated at 15°C in the dark in a humid chamber, which consisted of a plastic crisper with lid enclosing a plastic grid placed below four layers of moistened paper towels. After 3 weeks, inoculated fruits formed multiple sporodochium-like conidiomata developing in rings around the center of inoculation and producing numerous conidia of *P. vagabunda*. Spores of *N. kienholzii* and *P. vagabunda* were suspended in sterile water, and suspensions were adjusted to a final concentration of 1×10^5 conidia per ml, respectively. Twenty microliters of the inoculum suspension were used to inoculate wounded olive leaves using 10-leaf replicates. Leaves were wounded with a sterile needle as described above. The experiment was conducted twice using different isolates of each species (*N. kienholzii* isolates KARE943 and KARE1281, and *P. vagabunda* isolates KARE1943 and KARE1944). Because there were no significant differences between isolates belonging to the same pathogen species, data were combined. Factorial ANOVA was performed on the lesion diameter (mm) using cultivar and pathogen species as independent variables. Cultivar means were compared using Tukey's HSD test at $P = 0.05$.

Both the 2016 and 2017 leaf pathogenicity assays were conducted using 10 replicates per treatment organized in a completely randomized design. One leaf on each of 10 trees served as one replicate. Leaves inoculated with sterile water served as negative controls. Experiments concluded 3 months postinoculation in February 2017 and February 2018, respectively. Leaves were inspected for the development of necrotic lesions and taken to the laboratory for measurement of lesion diameter as well as attempts to recover the inoculated fungal isolates from the symptomatic tissues and fulfill Koch's postulates.

Olive shoot pathogenicity assays. The ability of *N. kienholzii* and *P. vagabunda* isolates to produce lesions in olive shoots was determined following two pathogenicity studies conducted in mature olive trees in the field in November 2016 and November 2017. The 'Arbequina', 'Arbosana', and 'Koroneiki' cultivars were used for these experiments. The full factorial combination of treatments was arranged in a completely randomized design with 10 replicates (olive trees) per treatment.

In the 2016 experiment, inoculation of shoots was conducted following artificial wounding that mimicked wounds caused by mechanical harvesters. The bark of shoots was sliced-opened using a sterile knife blade, creating a 5 × 10 mm wound that exposed the cambium. A 20-μl propagule suspension at 1×10^5 propagules per ml was placed immediately onto the surface of the wounds. Inocula of *N. kienholzii* and *P. vagabunda* consisted of conidia and mycelial fragments, respectively, and were produced as described in the 2016 leaf pathogenicity experiment above. This experiment was conducted using two representative isolates of each fungal species. Both assays consisted of two independent experiments using 1- to

2-year-old shoots on each of 10 olive trees (10 replicates) for each cultivar tested. Because both species were inoculated using different types of inoculum, the pathogenicity of each species was analyzed independently. Thus, ANOVAs were performed on the Log-transformed data and treatment means were compared using Tukey's HSD test at $P = 0.05$.

In the 2017 experiment, inoculation of olive shoots was conducted using mycelial plugs of the different isolates. The outer bark of 2-year-old shoots was removed using a sterile 5-mm-diameter cork borer. A 5-mm mycelial plug was excised from the margin of a 14-day-old OA colony and inserted into the wound, which was then sealed and wrapped with Parafilm. One branch on each of 10 trees was inoculated with one isolate, which accounted for 10 replicates. Additional branches were mock-inoculated with sterile OA plugs as above to serve as negative controls. Inoculated and mock-inoculated branches were collected after 3 months, and the length of lesions was measured above and below the point of inoculation for each shoot. Because there were no significant differences between isolates belonging to the same species, the data from the two isolates per species were combined for ANOVA. Log transformation was used to improve the normality and homogeneity of data. The transformed data were analyzed with ANOVA followed by Tukey's HSD test at $P = 0.05$.

Olive fruit pathogenicity assays. Pathogenicity studies were conducted to determine the ability of *N. kienholzii* and *P. vagabunda* to cause disease in fruits of 'Arbequina', 'Arbosana', and 'Koroneiki' cultivars. The experiment was conducted in the laboratory using detached fruits obtained from the field. Apparently healthy fruits were submerged for 3 min in 0.5% sodium hypochlorite solution, then rinsed twice in sterile distilled water and air-dried on sterile paper towels. The experiment was conducted on fruits wounded (via puncture) with a needle and inoculated with a 10-μl drop of a conidial suspension adjusted to 1×10^5 conidia per ml. Conidial suspensions were obtained for both *N. kienholzii* (KARE1066 and KARE1292) and *P. vagabunda* (KARE1943 and KARE1944) as described in the 2017 leaf inoculation experiment above. Sterile water was used for inoculation of the control fruits. Inoculated olives ($n = 6$ per treatment) were placed in moist chambers and incubated at 24°C. Inoculated and control fruits were assessed for fruit rot development after 14 days. The experiment was conducted twice using two different isolates for each fungal species. Because there were no significant differences between isolates belonging to the same species, the data from the two isolates per species were combined. Log transformation was used to improve the normality and homogeneity of these data. The transformed data were analyzed with ANOVA followed by Tukey's HSD test at $P = 0.05$.

Results

Disease symptoms and distribution. *N. kienholzii* and *P. vagabunda* leaf and twig lesions were detected mainly in the 'Arbosana' cultivar and to a lesser extent in the 'Arbequina' cultivar in SHD olive orchards in Glenn, San Joaquin, and Stanislaus counties. The disease was not found in the 'Koroneiki' cultivar. Symptoms were most visible in March, which corresponds to the early spring in California. Lesions on leaves were necrotic, circular to elongated, and usually occurred singly, ranging from 0.5 to 1 cm in diameter (Fig. 1A, top row, and B). Leaf lesions occurred at sites of tree injuries caused by mechanical harvesters and included abrasion sites where leaves rub against each other. Leaf lesions caused by *N. kienholzii* and *P. vagabunda* were similar but differed clearly from peacock spot lesions, as the former were necrotic, lacked the dark green halo typically associated with peacock spot (Fig. 1A, bottom row), and in general did not number more than one lesion per leaf (Fig. 1A, top row). Reddish-brown lesions on shoots and twigs and occasionally cankers in branches developed at wounds caused by mechanical harvesters (Fig. 1C, D, and E). Cankers in branches appeared as sunken lesions in the bark that elongated from the site of injury. The disease occasionally caused fruit spots in the 'Arbequina' cultivar and was visible in December on unharvested fruits remaining on trees (Fig. 1F). In severely affected orchards, 'Arbosana' trees showed

defoliation (Fig. 1G) and premature fruit drop. Finally, identification, using phylogenetic analyses, of all fungal isolates collected from twig and leaf lesions suggested that *N. kienholzii* was the most prevalent species associated in California (Table 1).

Phylogenetic analyses. PCR amplification of the ITS region, LSU, and *TUB2* generated 534–535, 841, and 880 bp fragments,

respectively. Alignments of the 109 isolates revealed a single haplotype of ITS and LSU for each of two separate species, and two haplotypes of *TUB2* were identified in each species; therefore the phylogenetic analyses were performed using a dataset including two isolates representing the two haplotypes across the three loci for each species as well as sequences from phylogenetic references.

Fig. 1. Symptoms on olive caused by *Neofabraea kienholzii* and *Phlyctema vagabunda* in California. **A**, Leaf lesions of *N. kienholzii* and *P. vagabunda* (top row) versus peacock spot symptoms on leaves (bottom row), caused by *Fusicladium oleagineum*. **B**, Leaf lesion of *N. kienholzii*. **C**, **D**, and **E**, Shoot lesions of *N. kienholzii* and *P. vagabunda*. **F**, Fruit spots in 'Arbequina' olives associated with *P. vagabunda*. **G**, Tree defoliation in a row of 'Arbosana' olives (left) heavily affected by *N. kienholzii* and *P. vagabunda* leaf and shoot lesions in comparison with a row of healthy 'Koroneiki' cultivar (right).

The best-fit model of nucleotide evolution for ML analyses were deduced based on the AIC (K2+G for ITS, K2+G+I for LSU, GTR+G for *TUB2*, and GTR+G+I for ITS+LSU+*TUB2*). The ITS dataset consisted of 559 characters (418 characters were constant, 46 characters were parsimony-uninformative, and 95 characters were parsimony informative). MP analysis produced 23 equally most parsimonious trees of 239 steps and a consistency index (CI), retention index (RI), and rescaled consistency index (RC) of 0.757, 0.838, and 0.634, respectively. The LSU dataset consisted of 841 characters (773 characters were constant, 22 characters were parsimony-uninformative, and 46 were parsimony informative). MP analysis produced 14 equally most parsimonious trees of 106 steps and a CI, RI, and RC of 0.726, 0.820, and 0.596, respectively. The *TUB2* dataset consisted of 817 characters (595 characters were constant, 28 characters were parsimony-uninformative, and 194 were parsimony informative). MP analysis produced six equally most parsimonious trees of 495 steps and a CI, RI, and RC of 0.574, 0.801, and 0.460, respectively. The ITS and LSU analyses were able to confidently ($\geq 88\%/97\%$ MP and ML bootstrap values, respectively) identify *P. vagabunda* isolates; however, support for *N. kienholzii* isolates was not realized in these analyses (Supplementary Figs. S1 and S2). Trees resulting from the analyses of *TUB2* strongly supported ($\geq 99\%/96\%$) the identification of Californian olive isolates as *N. kienholzii* and *P. vagabunda* (Supplementary Fig. S3). The three-gene dataset consisted of 2,216 characters (1,783 characters were constant, 98 characters were parsimony-uninformative, and 335 characters were parsimony informative). MP analysis produced 18 equally most parsimonious trees of 878 steps and a CI, RI, and RC of 0.621, 0.792, and 0.491, respectively. MP and ML results were similar to the *TUB2* locus whereby the combined three-locus dataset revealed that 67 Californian isolates clustered strongly (100%/100%) with the ex-type of *N. kienholzii* (CBS 126461) and 42 other Californian isolates clustered strongly (100%/100%) with the ex-type of *P. vagabunda* (CBS 304.62) (Fig. 2).

Morphological characterization. Examined fungi fit the description of *N. kienholzii* and *P. vagabunda* based on morphological characteristics. On PDA medium, *P. vagabunda* isolates were characterized by mostly round, even colonies with radial furrows and scarce aerial mycelium reaching 38 mm in 23 days. *P. vagabunda* colonies were white initially, turning pinkish in their center and brown in their periphery with time, and with compact, hyaline, and septate mycelium (Fig. 3A). On TA medium after 23 days, *P. vagabunda* produced 60-mm-diameter colonies that had a smooth and even margin with copious white aerial mycelium near the center with less aerial, light-brown mycelium at the colony fringe with many radial furrows. None of the isolates of *P. vagabunda* studied produced conidia on OA, PDA, or TA. However, conidia were produced successfully following inoculations of olive fruits as described previously. Conidiomata acervuloid to sporodochial producing conidia that were unicellular, hyaline, aseptate, fusiform to allantoid, with rounded and curved ends and averaged $21.6 \pm 0.4 \mu\text{m} \times 3.7 \pm 0.1 \mu\text{m}$ with a length-width ratio of 5.82 (Fig. 3B). *P. vagabunda* conidiomata also occurred in naturally infected leaves in the field (Fig. 3C).

N. kienholzii isolates produced 35-mm-diameter colonies with white to brownish mycelium after incubation on PDA medium after 23 days. PDA colonies expanded unevenly giving the colony a slightly torn appearance with submerged, brownish peripheral hyphae and copious white aerial hyphae near the center (Fig. 3D). On TA medium, *N. kienholzii* produced 55-mm-diameter, white, homogeneous wooly colonies with a smooth margin and even colony expansion after 23 days. Conidia were aseptate, ellipsoidal, slightly asymmetrical to slightly curved with sizes averaging $10.4 \pm 0.1 \mu\text{m} \times 3.6 \pm 0.1 \mu\text{m}$ and a length-width ratio of 2.85 (Fig. 3E and F). Naturally infected olive leaves produced many small, acervuloid to sporodochial conidiomata (Fig. 3F).

The optimal growth temperature was around 20°C for all three isolates of *N. kienholzii* tested and around 15°C for those of *P. vagabunda* (Fig. 4A and B). Mycelial growth rate averaged 2.53 mm per day for *N. kienholzii* and 3.02 mm per day for *P. vagabunda* cultured on TA medium for 21 days.

Pathogenicity tests. Olive leaf pathogenicity assays. In the 2016 experiment, no symptoms developed in leaves inoculated with the various fungal isolates in the absence of wounding. On the other hand, isolates KARE1066 or KARE1068 of *N. kienholzii* were pathogenic in leaves of the olive cultivars ‘Arbequina’, ‘Arbosana’, and ‘Koroneiki’ following wounding and inoculation with a conidial suspension. Isolates produced round to oval necrotic lesions averaging 6.37 mm in diameter after 3 months. No significant difference in aggressiveness, as measured by lesion diameter, was found between these isolates ($P = 0.698$). Likewise, the three olive cultivars did not differ significantly in their susceptibility to *N. kienholzii* isolates ($P = 0.438$). The interaction isolate-cultivar also was not significant ($P = 0.198$), revealing consistent cultivar responses to each isolate. In short, the *N. kienholzii* isolates were similarly pathogenic on the three olive cultivars that showed a similar phenotypic susceptibility (*data not shown*).

On the other hand, significant differences ($P = 0.011$) in susceptibility were observed among olive cultivars inoculated with a mycelial fragment suspension of *P. vagabunda*, with ‘Arbosana’ being more resistant than ‘Arbequina’ and ‘Koroneiki’; the latter two showed similar susceptibility to the pathogen. Finally, there was no significant difference in aggressiveness between *P. vagabunda* isolates KARE944 and KARE607 ($P = 0.487$), and no isolate-cultivar interaction was detected ($P = 0.776$); hence, aggressiveness data for both isolates were combined and analyzed (Fig. 5). Percent fungal recovery from developing lesions varied between 40 and 60% among all fungal isolates tested.

In the 2017 experiment, all isolates tested produced necrotic lesions in the wound-inoculated leaves. No symptoms were produced in the mock-inoculated control plants. Percent fungal recovery from developing lesions varied between 60 and 100% among all fungal isolates. Lesion size produced by isolates belonging to the same species did not differ significantly between isolates ($P > 0.05$). Accordingly, lesion diameter data were combined for isolates of the same species. Average lesion size measured 3 months after inoculations was 6.93 mm for *P. vagabunda* isolates and 6.66 mm for *N. kienholzii* isolates, respectively. There was a significant effect of the cultivar ($P < 0.001$) on lesion diameter but no significant difference in aggressiveness was detected between the two fungal species ($P = 0.638$). No interaction was found between the cultivar and fungal species ($P = 0.963$). With lesions averaging 6.8 mm and 7.2 mm in diameter, ‘Arbosana’ and ‘Koroneiki’ showed similar susceptibility, while ‘Arbequina’ was the least susceptible cultivar with lesions averaging 4.9 mm in diameter (Fig. 6).

Olive shoot pathogenicity assays. The experiment on olive shoots was conducted during two consecutive seasons using different fungal isolates and inoculation methods. Overall, the pathogen caused reddish-brown lesions in shoots that extended from the inoculation point and that were identical to those occurring in naturally infected twigs.

In 2016, the two *P. vagabunda* isolates, which were inoculated using a suspension of mycelial fragments, resulted in lesions similar to the lesions in the noninoculated control shoots, i.e., *P. vagabunda* isolates were not pathogenic in this case. Conversely, *N. kienholzii* isolates, which were inoculated using a conidial suspension, caused an average lesion size of 8.5 ± 0.9 mm across all cultivars. For *N. kienholzii*, ANOVA showed that there were significant ($P = 0.001$) differences in susceptibility to this pathogen among olive cultivars, whereas the isolate and the interaction of isolate and cultivar were not significant ($P > 0.05$). Thus, ‘Arbosana’ was the most susceptible cultivar (10.83 mm average lesion size) and ‘Koroneiki’ the most resistant (4.66 mm average lesion size). ‘Arbequina’ showed an intermediate resistance level (5.6 mm average lesion size).

In 2017, average lesion size produced across all cultivars and fungal isolates was 36 ± 5.4 mm. Combined ANOVA showed that only the olive cultivar had a significant effect ($P = 0.01$) on lesion length caused by the pathogens, while the effect of fungal species was not significant ($P > 0.05$). For this reason, fungal species was used as a block to compare the resistance of olive cultivars to the pathogens. According to these results, ‘Koroneiki’ (39.66 mm average lesion size) was significantly ($P < 0.05$) more susceptible than ‘Arbosana’ (35.16 mm average lesion size) and ‘Arbequina’ (34.62 mm average lesion size), which did not differ significantly between each other

(Fig. 7). All isolates tested were recovered from 100% of all inoculated shoots.

Olive fruit pathogenicity assays. All fungal isolates tested produced circular rot lesions extending from the inoculation points. Control fruits (noninoculated) showed no disease symptoms. There were significant effects on the lesion size associated with the fungal species ($P = 0.003$) and olive cultivar ($P = 0.0395$), but no interaction was found between species-cultivar ($P = 0.425$). In this assay, *N. kienholzii* was significantly more virulent than *P. vagabunda* in the

three evaluated olive cultivars. Also, the cultivar 'Koroneiki' (6.9 mm average lesion size) was significantly more susceptible than 'Arbequina' (5.8 mm average lesion size), whereas 'Arbosana' (6.3 mm average lesion size) showed an intermediate resistance (Fig. 8).

Discussion

N. kienholzii and *P. vagabunda* leaf and shoot lesions of olive are presently described and reported for the first time in California. This is the first report of *N. kienholzii* as a pathogen of olive trees, and it

Combined Multi-locus Dataset
ITS/LSU/TUB2
18 Trees
878 Steps
CI = 0.621
RI = 0.792
RC = 0.491

Fig. 2. One of 18 equally most parsimonious trees generated from maximum parsimony analysis of the three-gene (ITS+LSU+TUB2) combined dataset. Numbers in front and after the slash represent parsimony and likelihood bootstrap values from 1,000 replicates, respectively. Ex-type isolates are indicated in bold. Bar indicates the number of nucleotide changes.

was the most prevalent species associated with leaf and shoot lesions in California. Both fungal pathogens were identified using morphology and phylogenetic analyses, and both pathogens caused olive leaf and shoot lesions following wounding and inoculation with a conidial/mycelial suspension or mycelium plug. In this study, the disease was found only in SHD oil olive orchards in Glenn, San Joaquin,

and Stanislaus counties, although a previous study in California reported *P. vagabunda* from fruit spots in ‘Coratina’ and ‘Picholine’ cultivars in Sonoma County (Rooney-Latham et al. 2013).

Earlier reports in Italy and Spain have identified *P. vagabunda* (syn: *N. alba* and *N. vagabunda*) as the causal agent of leprosy fruit rot of olive (Petri 1915; Roca et al. 2007). The term “leprosy” has

Fig. 3. Asexual morphologies of *Phlyctema vagabunda* (A, B, C) and *Neofabraea kienholzii* (D, E, F). **A**, Colony of *P. vagabunda* cultured on PDA medium for 24 days at the laboratory's ambient conditions (24°C). **B**, Conidia of *P. vagabunda*. **C**, Conidiomata of *P. vagabunda* produced on a necrotic leaf lesion. **D**, Colony of *N. kienholzii* cultured on PDA medium for 24 days at the laboratory's ambient conditions (24°C). **E**, Conidia of *N. kienholzii*. **F**, Conidiomata of *N. kienholzii* produced on a necrotic leaf lesion. Scale bar in B and E = 10 μ m; in C and F = 1 mm.

been used initially to describe the olive fruit rot disease, characterized by round, dark brown, small necrotic lesions, slightly depressed and surrounded by a chlorotic halo (Roca et al. 2007). Twig, shoot, and leaf lesions caused by *P. vagabunda* have been recently reported in Spain (Romero et al. 2016, 2018). However, the same disease was reported earlier in Italy under anthracnose causing leaf and twig lesions in olive and was attributed to *Gloeosporium olivae* (Petri) Foschi (syn: *Cylindrosporium olivae* Petri) thus causing some confusion about the disease name and etiology (Foschi 1955). Currently, olive leprosy has been adopted to comprise fruit deformation and fruit rot as well as necrotic leaf and shoot lesions caused by *P. vagabunda* (Romero et al. 2018).

Today, oil olive orchards in California are more commonly cultivated in SHD hedgerows, at a density of 1,500 to 2,000 trees per hectare. This planting density is suitable for straddle harvesters, which work over the row by continuously beating the tree canopy, where most fruits are concentrated (Pérez-Ruiz et al. 2018; Tous et al. 2010). Over-the-row harvesters derived from grape harvesters vibrate the fruiting branches using shaking bars. Recent investigation has shown that nominal speed and beating frequency of such harvesters can increase canopy damage, particularly in olive cultivars less adapted to mechanical harvest (Pérez-Ruiz et al. 2018). In this study, field observations revealed that *N. kienholzii* and *P. vagabunda* leaf and shoot lesions were primarily associated with wounds sustained during mechanical harvest. Inoculation studies confirmed that wounds were required for the pathogens to successfully infect and cause lesions in leaves and shoots of olive. We hypothesized that the emergence of *N. kienholzii* and *P. vagabunda* pathogens in California olives is strongly correlated with the rise of SHD olive plantings

and the broad use of mechanical harvesters as it has been observed in Spain (Romero et al. 2018). Olive tree density affects disease development and generally higher density translates into higher disease incidence of olive anthracnose, caused by *Colletotrichum* spp. (Moral et al. 2009, 2012). High density plantings may facilitate transmission of infectious propagules through shorter dispersal distances between adjacent trees and create microclimatic conditions in the olive canopy more conducive to disease development. Mechanized cultivation operations also create more wounds in SHD, with wounds serving as infection courts for pathogens such as *Botryosphaeriaceae* spp. and *Pseudomonas savastanoi* pv. *savastanoi* (Moral et al. 2009, 2012). This production system also has led to increased infections by *P. savastanoi* pv. *savastanoi* in California (Nguyen et al. 2017). The development of SHD olive orchards most likely has facilitated the emergence of *N. kienholzii* and *P. vagabunda* leaf and shoot lesions in California.

Pathogenicity studies indicated that leaves and twigs of 'Arbequina', 'Arbosana', and 'Koroneiki' cultivars were susceptible to *N. kienholzii* and *P. vagabunda* following wounding and artificial inoculation. In contrast, mainly leaves and twigs of the 'Arbosana' cultivar were affected in the field, while leaf and twig lesions were sporadic in 'Arbequina' and not found in 'Koroneiki' cultivars. The abundance of *N. kienholzii* and *P. vagabunda* leaf and shoot lesions in 'Arbosana' olives in the field suggests that this cultivar might be more susceptible to wounding and abrasion injuries during harvest than the cultivars 'Arbequina' and 'Koroneiki'. Pathogenicity studies also revealed that different tissues responded differently in their susceptibility to *N. kienholzii* and *P. vagabunda*.

Fig. 4. Effect of temperature on the mycelial growth of three isolates of *Phlyctema vagabunda* (KARE1288, KARE1943, and KARE1944) (A) and *Neofabraea kienholzii* (KARE943, KARE1065, and KARE1281) (B) cultured on tomato agar for 21 days in the dark.

The genus *Neofabraea* and related anamorphs include important plant pathogens of tree crops, in particular pome fruits. *Neofabraea* species, including *N. kienholzii*, *N. malicorticis* (Cordley) H.S. Jacks., *N. perennans* Kienholz, and *P. vagabunda*, are responsible for the bull's eye rot, a severe postharvest decay of apple and pear fruits in the Pacific Northwest USA (de Jong et al. 2001; Henriquez et al. 2004, 2006; Spotts et al. 2009; Verkley 1999). Recently, increasing reports of bull's eye rot in pome fruits have emerged worldwide including in Australia (Cunnington 2004), Chile (Henriquez 2005; Soto-Alvear et al. 2013), Italy (Cameldi et al. 2017), and the Netherlands (Wenneker et al. 2017). *Neofabraea* species also cause canker diseases in pome fruits (Aguilar et al. 2019; Garipey et al. 2005; Henriquez et al. 2006), bark lesions on poplars (Kasanen et al. 2002), and coin canker of ash (*Fraxinus*; Oleaceae) in the northern United States and Canada (Putnam and Adams 2005). In this study, we report for first time the species *N. kienholzii* affecting olive. Thus far, *N. kienholzii* has been reported from pome fruits in Canada, Europe, and the United States (Cunnington 2004; de Jong et al. 2001; Henriquez et al. 2004; Michalecka et al. 2016; Spotts et al. 2009) and the ornamental plant *Aucuba japonica* (Chen et al. 2016). A recent study in California reported *P. vagabunda* (syn: *N. alba*) causing

branch canker and dieback of apple in Sonoma County (Rooney-Latham and Soriano 2016). The same pathogen was reported from symptomatic pear fruits in California (Henriquez et al. 2004). Despite distinct climatic requirements for fruit production, pear and apple orchards have been cultivated in areas where SHD oil olive orchards are planted in California. This raises the question about the possible role of apple and pear trees to serve as inoculum reservoirs for this emerging disease of olive. Future studies should investigate the occurrence of *P. vagabunda* and *N. kienholzii* from these different hosts to address this hypothesis.

Several studies have suggested that *P. vagabunda* can survive on dead bark tissues of apple, pear, and olive trees serving as inoculum for new infections (Verkley 1999). In Michigan, this fungus has been reported to cause the coin canker disease of ash (Rossman et al. 2002). Ash trees are frequently found in riparian areas near olive orchards in the San Joaquin Valley of California. This information is important for the identification of primary inoculum sources that eventually invade susceptible olive orchards leading to epidemics in a conducive environment. The causal agents of bull's eye rot of pome fruits can vary in virulence (Kienholz 1939) and respond differently to chemical control measures (Spotts et al. 2009). The same could also be true for *N. kienholzii* and *P. vagabunda* causing leaf and shoot lesions of oil olives in California. Therefore, it is critical to

Fig. 5. Average lesion diameter (mm) produced by *Phlyctema vagabunda* in leaves of 'Arbequina', 'Arbosana', and 'Koroneiki' olive cultivars (2016 experiment). Columns topped with different letters indicate treatment means that are significantly different according to Tukey's HSD test ($P < 0.05$). Error bars represent the standard error of the mean.

Fig. 6. Average lesion diameter (mm) produced by *Neofabraea kienholzii* and *Phlyctema vagabunda* in leaves of 'Arbequina', 'Arbosana', and 'Koroneiki' olive cultivars (2017 experiments). No significant ($P = 0.638$) differences were found between fungal species. Columns topped with different letters indicate treatment cultivars that are significantly different according to Tukey's HSD test ($P < 0.05$). Error bars represent the standard error of the mean.

Fig. 7. Average lesion length (mm) produced by *Neofabraea kienholzii* and *Phlyctema vagabunda* in shoots of 'Arbequina', 'Arbosana', and 'Koroneiki' olive cultivars (2017 experiments). No significant ($P = 0.658$) differences were found between fungal species. Columns topped with different letters indicate treatment cultivars that are significantly different according to Tukey's HSD test ($P < 0.05$). Error bars represent the standard error of the mean.

Fig. 8. Average lesion diameter (mm) produced by *Phlyctema vagabunda* and *Neofabraea kienholzii* in fruits of 'Arbequina', 'Arbosana', and 'Koroneiki' olive cultivars (2017 experiments). Across the three cultivars, *N. kienholzii* was significantly ($P < 0.05$) more virulent than *P. vagabunda*. Columns topped with different letters indicate cultivar treatments that are significantly different according to Tukey's HSD test ($P < 0.05$). Error bars represent the standard error of the mean.

monitor the prevalence of the pathogens and changes in their populations as this will affect management strategies of this new destructive disease in SHD California olive orchards. In this study, surveys revealed that *N. kienholzii* prevails in abundance over *P. vagabunda* in diseased leaves and twigs of olive in California.

Mechanical harvest of oil olives combined with mild temperatures and heavy rainfalls are factors that most likely have contributed to the emergence of *N. kienholzii* and *P. vagabunda* leaf and shoot lesions in SHD orchards. In California, oil olive harvest occurs generally mid- to late November, which also corresponds to the start of the rainy season. The occurrence of rain following recent wounding of shoots, twigs, and leaves probably creates ideal conditions for *N. kienholzii* and *P. vagabunda* pathogens to infect olive trees. Similarly, with optimum temperatures for growth ranging between 15 and 20°C, respectively, *P. vagabunda* and *N. kienholzii* seem well adapted to cause diseases during the fall season in California, when daytime temperatures remain mild. Field observations of seasonal symptom progression suggest that these pathogens may remain latent during the winter months, as leaf and shoot lesions mainly become apparent in March.

As mechanized practices and SHD production systems become increasingly employed in many olive production areas worldwide, we expect that *N. kienholzii* and *P. vagabunda* leaf and shoot lesions will continue to rise significantly in the future. Romero et al. (2018) reported dramatic increase of Phlyctema disease in southern Spain and Portugal in recent years, as these olive industries continue to expand and intensify production practices with the introduction of mechanized pruning and harvesting.

In California, copper is the only fungicidal and bactericidal compound currently registered by the state for use on olive. It is often used as a foliar treatment for the control of olive knot. It is generally applied after harvest and following natural leaf drop in the spring (Teviotdale and Krueger 2004). Similarly, copper has also been employed for the management of peacock leaf spot (olive leaf spot) and *Cercospora* leaf spot, and it is typically applied several times a year near rain events. The broad use of copper in California olive orchards has apparently not prevented the emergence of *N. kienholzii* and *P. vagabunda* leaf and shoot lesions, and we suspect that this compound is inefficient for the control of these diseases. Similarly, Romero et al. (2015) reported little to no efficacy of copper against *P. vagabunda* in Spain. Ongoing studies in our laboratory are investigating the efficacy of various fungicides to control and limit further spread of these emerging diseases.

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