Etiology of Branch Dieback and Shoot Blight of English Walnut Caused by Botryosphaeriaceae and *Diaporthe* Species in Southern Spain

Ana López-Moral,¹ María Lovera,² María del Carmen Raya,¹ Nerea Cortés-Cosano,¹ Octavio Arquerro,² Antonio Trapero,¹ and Carlos Agustí-Brisach^{1,†}

¹ Departamento de Agronomía, ETSIAM, Universidad de Córdoba, Campus de Rabanales, 14071 Córdoba, Spain

² Departamento de Fruticultura Mediterránea, IFAPA, 14004 Córdoba, Spain

Abstract

English walnut (*Juglans regia* L.) is considered an economically important fruit crop worldwide. In Spain, little attention has been given to walnut diseases owing to the minor economic importance of the walnut crop in the country until recently. In 2017, typical symptoms of branch dieback and shoot blight of English walnut were observed in southern Spain. From 2017 to 2018, 10 commercial walnut orchards showing disease symptoms were surveyed. Botryosphaeriaceae and *Diaporthe* fungi were consistently isolated from affected shoots. *Cytospora* isolates were also recovered with minor relevance. Representative isolates of each fungal group were characterized based on colony and conidial morphology, optimum growth temperature, and comparison of DNA sequence data from the internal transcribed spacer, elongation factor 1- α , and β -tubulin genomic areas. Pathogenicity tests were performed on detached and attached shoots and on detached fruit by inoculating them with mycelial plugs. Botryosphaeriaceae and *Diaporthe* isolates had higher optimum

growth temperatures (≈ 25 to 27°C) than *Cytospora* sp. (19.5°C). The following species were identified: Botryosphaeriaceae: *Botryosphaeria dothidea*, *Diplodia seriata*, *Dothiorella sarmentorum*, *Dothiorella* sp., *Neofusicoccum mediterraneum*, and *N. parvum*; *Diaporthe*: *Diaporthe neotheicola*, *Dia. rhusicola*, *Diaporthe* sp., and *Phomopsis amygdali*; and *Cytospora* sp. Botryosphaeriaceae isolates were the most aggressive fungi to walnut in all tissues evaluated, followed by *Diaporthe* isolates and *Cytospora* sp. *N. parvum* was the most virulent among the remaining species tested in any of the tissues evaluated, followed by *B. dothidea* or *N. mediterraneum*. This work is the first report to identify the fungal species causing this complex disease of English walnut in Spain and Europe.

Keywords: canker diseases, diagnosis, identification, *Juglans regia*, pathogenicity, tree nuts

English walnut (*Juglans regia* L.) is considered an economically important tree cultivated for nuts and wood production worldwide. China and the United States are the main producer countries of nuts, with 43% (880,000 t) and 30% (611,280 t) of the total fruit production in the world, respectively (International Nut and Dried Fruit Council 2017; López-Larrinaga et al. 2017). Nevertheless, despite the favorable edaphoclimatic and orographic conditions of the Iberian Peninsula's lands for this crop, to date the economic impact of the English walnut crop in Spain has been low (Lovera et al. 2017a, 2017b). Indeed, the total growing surface of this nut crop in Spain is only approximately 10,367 ha, with 15,744 t of nut production. The main walnut growing region in this country is Andalusia (southern Spain), with approximately 2,218 ha of cultivated area and 2,538 t of nut production, followed by Extremadura (west-southern Spain; 1,645 ha, 2,414 t) and Castilla La Mancha (central Spain; 1,473 ha, 1,305 t) (López-Larrinaga et al. 2017; Ministerio de Agricultura, Pesca y Alimentación 2017). In addition, the European Union has a high deficit in nut production, and its market depends markedly on international imports. Additional advantages of this nut crop, such as its high rentability and the high level of mechanization of the cultural practices available for walnut growers,

implicate the English walnut as a potential alternative nut crop in Spain (Lovera et al. 2017a, 2017b). In fact, the Spanish walnut growing surface increased by 13% of the cultivated surface between 2013 and 2016 (López-Larrinaga et al. 2017).

In Spain, little attention has been given to research on walnut diseases owing to the minor economic importance of this nut crop in the country until recently. Likewise, fruit diseases are traditionally considered the most important disorders that cause economic yield losses in this crop. Studies conducted recently in southern Spain by Lovera et al. (2017a, 2017b) indicated that the bacterium *Xanthomonas arboricola* pv. *juglandis* is the main causal agent of walnut blight and is associated with many other bacteria and fungi that can coinfect the affected nuts, increasing the disease severity. Nevertheless, since late spring to summer 2017, typical symptoms of shoot blight including spur, twig, bud, and leaf blight and branch dieback with wood canker formation and internal wood discoloration have been observed in commercial English walnut orchards in southern Spain. Although the disease has been previously described in California, causing serious damage to the commercial English walnut orchards located in the main nut growing counties in San Joaquín and Sacramento Valleys (Chen et al. 2014; Michailides and Hasey 2010), the etiology and biology of the disease are still uncertain in other walnut growing regions of the world, such as Spain.

Previous studies revealed that Botryosphaeriaceae and *Diaporthe* (syn. *Phomopsis*) are the main fungal species associated with the symptoms described above in English walnut (Chen et al. 2014; Li et al. 2015; Michailides and Hasey 2010). It is well known that Botryosphaeriaceae and *Diaporthe* fungi are widespread worldwide, causing many important diseases of woody crops, including fruit rots, branch dieback, shoot blight, and stem cankers, among other symptoms (Guarnaccia et al. 2018; Phillips et al. 2008). To date, the following 12 fungal species belonging to these two groups of fungi have been described as causing branch dieback and shoot blight of English walnut in California: (i) Botryosphaeriaceae: *Botryosphaeria dothidea*, *Diplodia mutila*, *D. seriata*, *Dothiorella iberica*, *Lasiopodia citricola*, *Neofusicoccum mediterraneum*, *N. nonquasitum*,

[†]Corresponding author: C. Agustí-Brisach; cagusti@uco.es

Funding: This research was funded by the Junta de Andalucía (project 'PPTRATRA-2016.00.6. Transforma de Fruticultura Mediterránea' from the Andalusian Institute for Research and Formation in Agriculture and Fishery, IFAPA). C. Agustí-Brisach is the holder of a 'Juan de la Cierva-Incorporación' fellowship funded by the Spanish Ministry of Science, Innovation and Universities (MICINN).

The author(s) declare no conflict of interest.

Accepted for publication 1 August 2019.

N. parvum, *N. vitifusiforme*, and *Neoscytalidium dimidiatum*; (ii) *Diaporthe*: *Diaporthe neotheicola* (syn. *Dia. foeniculacea*, *Dia. foeniculina*) and *Dia. rhusicola* (Chen et al. 2014; Michailides and Hasey 2010; Trouillas et al. 2010). Additionally, *B. dothidea* and *Lasiodiplodia pseudotheobromae* have also been associated with the disease in China (Li et al. 2015) and *D. mutila* in Chile (Díaz et al. 2018). *Juglans juglandina*, belonging to *Diaporthales*, has been identified as a causal agent of cankers in *J. nigra* and *J. regia*. The asexual morph of this species is common in Asia and Europe (Voglmayr et al. 2017).

Species belonging to the genus *Cytospora* have also been described as destructive canker pathogens that cause dieback in a broad range of woody plant species, including *Juglans* and *Prunus* spp. (Lawrence et al. 2017). In China, *Cytospora atrocirrhatta*, *C. chrysosperma*, *C. gigalocus*, *C. nivea*, and *C. sacculus* were identified as causal agents of branch dieback and cankers in English walnut (Fan et al. 2015; Zhao et al. 2018).

Finally, other canker diseases, such as thousand cankers disease, have also been described as affecting *Juglans* species in forests. Wilt-ing, yellowing of leaves, and branch dieback are the typical symptoms of the disease. Thousand cankers disease is caused by the fungus *Geosmithia morbida*, which is widely distributed across the western United States, and it is vectored primarily by the walnut twig beetle *Pityophthorus juglandis* (Daniels et al. 2016).

With respect to canker diseases in Spain, many *Botryosphaeria*-ceae species have been previously reported to be associated with branch dieback and general decline in the main woody crops growing in this country, such as almond (Gramaje et al. 2012; Olmo et al. 2017), grapevine (Agustí-Brisach et al. 2019a; Armengol et al. 2001; Aroca et al. 2006, 2008; Martin and Cobos 2007), and olive (Agustí-Brisach et al. 2017; Moral et al. 2010, 2017), as well as in forests, such as *Quercus* spp. (Sánchez et al. 2003). Concerning *Diaporthe* species, *Phomopsis amygdali* has been described as causing cankers and wood decay of almond in Spain (Gramaje et al. 2012; Olmo et al. 2017). Recently, Guarnaccia et al. (2018) conducted a broad survey of grapevines in Europe to understand the *Diaporthe* diversity affecting this crop. In particular, the following *Diaporthe* species have been associated with *Phomopsis* cane and leaf spot of grapevine in Spain: *Dia. ambigua*, *Dia. ampelina*, *Dia. baccae*, *Dia. eres*, *Dia. hispaniae*, *Dia. hungariae*, and *Dia. rudis* (Guarnaccia et al. 2018). In Spain, several *Cytospora* spp. have been described causing cankers in woody crops and forests (Melgarejo et al. 2010; Sánchez-Solana et al. 2018). Recently, *Cytospora pruinosa* has been reported for the first time in this country as causing branch dieback of olive with low severity (Moral et al. 2017). However, this genus has not been extensively studied in tree nuts in Spain.

Botryosphaeriaceae, *Cytospora*, and *Diaporthe* fungi are considered saprophytes and endophytes, and they are able to survive on dead or inactive plant tissues (i.e., bark or wood of trees). Many of those pathogenic species have opportunistic behavior, and they are considered facultative wound parasites that attack weakened hosts (Agustí-Brisach et al. 2019b; Gomes et al. 2013; Guarnaccia et al. 2018; Lawrence et al. 2017; Phillips et al. 2008; Úrbez-Torres 2011; Úrbez-Torres and Gubler 2009; Úrbez-Torres et al. 2006,

2013). These pathogens develop fruiting bodies (pycnidia) on dead tissues and produce viable spores over long periods of time, providing the spore source to infect plants through natural (i.e., fruit abscission point, leaf scars, etc.) or artificial wounds (i.e., pruning wounds) (Michailides and Hasey 2010; Moral et al. 2019; Rolshausen et al. 2010; Úrbez-Torres et al. 2013). In addition, coinfections between *Botryosphaeriaceae* and *Diaporthe* species could cause a possible retarding or antagonistic effect on infection and disease development. In fact, studies conducted in California by Agustí-Brisach et al. (2019b) showed a significant delay in lesion development in shoots and hulls of English walnut when *Dia. rhusicola* was inoculated 4 days before *N. mediterraneum* with respect to the other interaction treatments evaluated.

Although studies on the etiology and epidemiology of branch dieback and shoot blight of English walnut have been conducted recently in the two main growing regions of this crop in the world (United States and China), the etiology of the disease in Spain has not yet been studied. Moreover, the extension of this nut crop to growing areas with favorable environmental conditions for disease development, such as southern Spain, has contributed to a major incidence of this complex disease during the last few years. Studies on etiology are essential for a good disease diagnosis to develop future research on the epidemiology and control of the disease. Therefore, the objectives of this work were to (i) identify the different fungal species associated with branch dieback and shoot blight of English walnut by morphology and molecular phylogeny and (ii) evaluate their pathogenicity to walnut shoots and green fruit (epicarp) under laboratory and field conditions.

Materials and Methods

Field surveys and fungal isolation. From spring 2017 to summer 2018, field surveys were conducted to study the occurrence of branch dieback and shoot blight of English walnut in southern Spain. Ten commercial walnut orchards belonging to Andalusia (Almería, Granada, Málaga, and Sevilla provinces) and Extremadura regions (Badajoz Province) were surveyed. These fields grew the English walnut cultivars (cvs.) Chandler, Hartley, Serr, Tulare, or Vina, with ages ranging from 12 to 35 years (Table 1). In each orchard, at least three walnut trees showing symptoms of branch dieback and shoot blight were examined, and the affected branches and shoots were cut. Wood samples showing internal discoloration and/or pycnidia on the bark were collected, placed in plastic bags, and kept at 4°C until isolations were made in the laboratory.

Once in the laboratory, the outer bark of affected wood samples was removed. Subsequently, small wood fragments from the margin of the affected area of each symptomatic branch were surface disinfested by dipping the fragments into a commercial bleach (Cl at 50 g/liter) solution at 10% (vol/vol) in sterile water for 2 min, air dried on sterile filter paper for 15 min, and plated on potato dextrose agar (PDA; Difco Laboratories, Le Pont de Claix, France) acidified with lactic acid (2.5 ml of 25% [vol/vol] per liter of medium) (APDA). When fungal fruiting structures were present on the affected tissues, they were removed carefully with a sterile needle and plated directly

Table 1. Commercial walnut orchards showing branch dieback and shoot blight surveyed between 2017 and 2018 in southern Spain

Field	Location (municipality/province/region)	Walnut cultivar	Age of trees (years)	Date of survey (month/day/year)
1	Antequera, Málaga, Andalucía	Vina	12	04/11/2017
2	Benavides, Badajoz, Extremadura	Tulare	15	07/14/2017
3	Benavides, Badajoz, Extremadura	Serr	15	07/14/2017
4	Badajoz, Badajoz, Extremadura	Chandler	35	07/10/2017
5	Guadix, Granada, Andalucía	Chandler	14	07/26/2017 07/19/2018
6	Chirivel, Almería, Andalucía	Chandler	15	07/27/2017
7	Alcalá del Río, Sevilla, Andalucía	Chandler	16	10/18/2017
8	Badajoz, Badajoz, Extremadura	Hartley	35	11/03/2017
9	Badajoz, Badajoz, Extremadura	Serr	30	11/03/2017
10	Potted plants of commercial nursery	Vina	2	07/12/2018

onto APDA. A total of 630 wood pieces obtained from the margin of the affected tissues or fruiting bodies were plated on Petri dishes for fungal isolation (inoculation points on Petri dishes: 10 orchards $\times$ 3 walnut trees $\times$ 3 Petri dishes $\times$ 7 wood pieces or fruiting bodies per Petri dish). Petri dishes were incubated for 5 to 14 days at 25°C in darkness until colonies were large enough to be examined. To obtain pure cultures, hyphal tips from the margin of the fungal colonies were transferred to PDA and incubated as described above. Based on our previous experience in the diagnosis of fungal trunk pathogens, and according to colony color and mycelial growth development, fungal isolates from each orchard were grouped into three fungal groups: Botryosphaeriaceae (light to dark gray, fast-growing mycelium), *Diaporthe* (beige-white, middle- to fast-growing mycelium) or *Cytospora* (beige to olive gray, middle- to slow-growing mycelium). Pycnidia production was induced when it was necessary to observe conidia (see the protocol below). These morphological observations were helpful in selecting 39 representative isolates that were used for further analysis (Table 2). To obtain pure cultures, all the isolates were single-spored prior to morphological and molecular identification using the serial dilution method described by Dhingra and Sinclair (1995). They were transferred to sterile plastic tubes filled with PDA, and sterile paraffin oil (Panreac Química SA, Barcelona, Spain) was added after colonization for long-term storage. The studied isolates are registered and maintained at 4°C in darkness in the collection of the Department of Agronomy at the University of Córdoba, Spain.

Fungal colony, conidia, and pycnidia morphology. Based on colony characteristics (texture, density, color, zonation, margin, and growth rate), isolates collected from walnut trees showing branch dieback were first identified at the family or genus level. According to this identification, 10 representative isolates (Table 2) of the different types of colonies observed were used to study colony morphology after growing the isolates on PDA at 25 $\pm$ 2°C in darkness. Botryosphaeriaceae, *Cytospora*, and *Diaporthe* fungi were first identified at the genus level by colony morphology when grown on PDA (Gomes et al. 2013; Phillips et al. 2013; Norphanphoun et al. 2017). To induce pycnidia production for conidium and pycnidium characterization, all fungal isolates were grown on pistachio (*Pistacia vera* L.) leaf agar (LA) (Agustí-Brisach et al. 2019b; Chen et al. 2014) at 25°C for 3 weeks under continuous fluorescent light.

Characteristics of mycelia were recorded after 4 to 10 days (depending on the mycelial growth development of each isolate) of incubation at 25°C in darkness (Barnett and Hunter 1998), and color was determined with a color scale (Kornerup and Wanscher 1963). For conidial measurements, conidial masses from pycnidia obtained from the surface of LA were placed on slides with a drop of 0.01% acid fuchsin in lactoglycerol (1:2:1 lactic acid/glycerol/water) and covered with a coverslip. Fungal structures were observed and measured at a $\times$ 400 magnification or at a $\times$ 1,000 magnification under the oil-immersion lens, depending on the conidial size, using a Nikon Eclipse 80i microscope (Nikon, Tokyo, Japan). Pycnidia were measured directly from the surface of LA at a $\times$ 20–7.5 magnification using a Nikon SMZ18 binocular. For each isolate, 30 measurements were obtained for each structure. The average length and width were calculated. The length/width ratio was also obtained for conidia.

Effect of temperature on mycelial growth. The 10 representative isolates selected in this study for morphological characterization (Table 2) were grown on PDA at 25°C in darkness for 10 days. Mycelial plugs (7.5-mm diameter) obtained from the margins of actively growing colonies were placed in the center of Petri dishes filled with PDA. Petri dishes were incubated at 5, 10, 15, 20, 25, 30, and 35°C in darkness. The largest and smallest diameters of the colonies were measured with a digital scale ruler from 4 to 10 days after inoculation, depending on the mycelial growth development of each isolate. Mean data were converted to radial growth rate (mm/day). There were four replicate Petri dishes per isolate and temperature combination, and the experiment was conducted twice.

For each isolate, a nonlinear adjustment of the data was applied using the generalized Analytics Beta model (Hau and Kranz 1990) to evaluate the variation in mycelial growth rate over temperature

(López-Moral et al. 2017). For each isolate, the average growth rates at each temperature were adjusted to a regression curve to estimate the optimum growth temperature and the maximum growth rate (MGR). Data were tested for normality and homogeneity of variances, and ANOVA was performed with the optimum growth temperature or the MGR as dependent variables and isolate as the independent variable. For each variable, isolate means were compared according to Tukey's honestly significant difference (HSD) test at α = 0.05 (Steel and Torrie 1985). Data from this experiment were analyzed using Statistix 10 software (Analytical Software 2013).

Molecular characterization. DNA extraction. Genomic DNA was obtained from the active mycelium of the 39 fungal isolates belonging to the Botryosphaeriaceae, *Cytospora*, and *Diaporthe* fungi included in this study (Table 2) that were growing on PDA as described above. Mycelial tissues were ground using the FastPrep-24 grinder machine (MP Biomedicals, Santa Ana, CA). Subsequently, DNA extractions were performed using the E.Z.N.A. Fungal DNA Kit (OMEGA BioTek, Norcross, GA) following the manufacturer's instructions. A MaestroNano spectrophotometer (MaestroGen, Taiwan) was used to determine the concentration and purity of the extracted DNA.

PCR analysis and sequencing. For all of the isolates, the 5.8S nuclear ribosomal gene with two flanking internal transcribed spacers (ITS) and the genomic areas elongation factor 1- α (EF) and β -tubulin (TUB) were amplified. ITS was amplified using the primer pair ITS4/ITS5 (White et al. 1990), and EF and TUB were amplified with the primer pairs EF1-728F/EF1-986R (Carbone and Kohn 1999) and T1 (O'Donnell and Cigelnik 1997)/Bt2b (Glass and Donaldson 1995), respectively. The PCRs were performed in a total volume of 25 μ l containing the following mixture: 20 ng of genomic DNA, 5 μ l of 5 $\times$ My Taq Reaction Buffer, and 0.13 μ l of My Taq DNA Polymerase (Bioline, Memphis, TN). Additionally, 0.2 μ M (each) primer was used for the EF and TUB PCRs, whereas for the ITS region PCR, 0.4 μ M (each) primer was added. A negative control was included in all PCRs using ultrapure water instead of DNA. The PCR cycling programs were conducted as follows: for ITS, initial denaturation at 94°C for 3 min, followed by 30 cycles of 94°C for 30 s, 48°C for 30 s, and 72°C for 1 min, and a final extension at 72°C for 10 min; for EF, initial denaturation at 94°C for 3 min, followed by 35 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 45 s, and a final extension at 72°C for 10 min; and for TUB, initial denaturation at 95°C for 3 min, followed by 35 cycles of 94°C for 15 s, 58°C for 15 s, and 72°C for 1 min, and a final extension at 72°C for 7 min. All PCRs were carried out in a MyCycler Thermal Cycler (Bio-Rad, Hercules, CA). Amplification products were checked by electrophoresis with a 1.5% (w/v) agarose gel stained with RedSafe (Intron Biotechnology, Seongnam-Si, Korea) and visualized under ultraviolet light. A 100-bp DNA Ladder-GTP (gTPbio, C. Viral, Sevilla, Spain) was used as a molecular weight marker. Subsequently, the PCR products were purified using the MEGAquick-spin Total Fragment DNA Purification kit (Intron Biotechnology) following the manufacturer's instructions. The resulting amplicons were sequenced in both directions by the Central Service Support Research of the University of Córdoba (Spain).

Phylogenetic analysis. Consensus sequences were obtained from DNA sequences generated with forward and reverse primers using SeqMan software (DNASTART Lasergen SeqMan version 7.0.0, Madison, WI) and were compiled into a single FASTA file format. To determine whether the sequence datasets (ITS/EF/TUB) were congruent and combinable, a partition-homogeneity test was carried out by PAUP version 4.0b10 (Swofford 2003), and neighbor-joining analysis was performed individually for each locus (data not shown). The neighbor-joining analysis was conducted using the maximum composite likelihood method and 2,000 bootstrap replications, and genetic distances were calculated using the Kimura two-parameter mode. The partition-homogeneity test (P = 0.39) was not significant for the combined ITS/EF/TUB data set; consequently, they could be combined. Moreover, tree topologies of 70% reciprocal bootstrap generated individually for each locus were compared visually, and

Table 2. Fungal isolates used in the phylogenetic analysis and their corresponding GenBank accession numbers

Species	Isolate ^x	Host/cultivar	Origin	Collector	Date ^y	GenBank accession number ^z		
						ITS	EF	TUB
Botryosphaeriaceae analyses								
<i>Botryosphaeria dothidea</i>	ColPat-443*	<i>Juglans regia</i> cv. Tulare	Benavides, Badajoz, Spain	C. Agustí-Brisach & A. Trapero	07/14/2017	MK431835	MK461889	MK522082
	ColPat-446	<i>J. regia</i> cv. Serr	Benavides, Badajoz, Spain	C. Agustí-Brisach & A. Trapero	07/14/2017	MK431836	MK461890	MK522083
	ColPat-530	<i>J. regia</i> cv. Chandler	Alcalá del Río, Sevilla, Spain	C. Agustí-Brisach & A. Trapero	10/18/2017	MK431843	MK461897	MK522084
	ColPat-553	<i>J. regia</i> cv. Hartley	Badajoz, Spain	C. Agustí-Brisach & A. Trapero	11/03/2017	MK431844	MK461898	MK522085
	CMW8000 ^T	<i>Prunus</i> sp.	Crocifisso, Switzerland	B. Slippers	n/d	AY236949	AY236898	AY236927
<i>Diplodia juglandis</i>	6I19	<i>J. regia</i>	Yuba, CA	T. J. Michailides	09/16/2011	KF778786	KF778976	KF778881
	CBS 188.87	<i>J. regia</i>	France	n/d	n/d	EU673316	EU673283	EU673119
<i>Diplodia mutila</i>	CBS 112553 ^T	<i>Vitis vinifera</i>	Montemor-o-Novo, Portugal	A. J. L. Phillips	n/d	AY259093	AY573219	DQ458850
<i>Diplodia seriata</i>	6B99	<i>J. regia</i>	Yolo, CA	T. J. Michailides	05/31/2011	KF778791	KF778981	KF778886
	ColPat-366	<i>J. regia</i> cv. Vina	Antequera, Málaga, Spain	C. Agustí-Brisach & A. Trapero	04/11/2017	MK431829	MK461883	MK522086
	ColPat-367	<i>J. regia</i> cv. Vina	Antequera, Málaga, Spain	C. Agustí-Brisach & A. Trapero	04/11/2017	MK431830	MK461884	MK522087
	ColPat-557*	<i>J. regia</i> cv. Serr	Badajoz, Spain	C. Agustí-Brisach & A. Trapero	11/03/2017	MK431847	MK461901	MK522088
	CBS 112555 ^T	<i>Vitis vinifera</i>	Montemor-o-Novo, Portugal	A. J. L. Phillips	n/d	AY259094	AY573220	DQ458856
<i>Dothiorella iberica</i>	3H18	<i>J. regia</i>	Parlier, Fresno, CA	T. J. Michailides	01/15/2004	KF778796	KF778986	KF778891
	CBS 115041 ^T	<i>Quercus ilex</i>	Spain	J. Luque	n/d	AY573202	AY573222	EU673096
<i>Dothiorella omnivora</i>	5G97	<i>J. regia</i>	Davis, Yolo, CA	T. J. Michailides	12/13/2010	KF778808	KF778998	KF778903
	CBS 392.80 ^T	n/d	France	n/d	n/d	KX464133	KX464626	KX464897
<i>Dothiorella sarmentorum</i>	ColPat-469	<i>J. regia</i> cv. Chandler	Chirivel, Almería, Spain	C. Agustí-Brisach & A. Trapero	07/27/2017	MK431838	MK461892	MK522089
	ColPat-471*	<i>J. regia</i> cv. Chandler	Chirivel, Almería, Spain	C. Agustí-Brisach & A. Trapero	07/27/2017	MK431840	MK461894	MK522090
	IMI 63581b ^T	<i>Ulmus</i> sp.	Warwickshire, U.K.	E. A. Ellis	n/d	AY573212	AY573235	EU673102
	CBS 115038	<i>Malus pumila</i>	Delft, The Netherlands	A. J. L. Phillips	n/d	AY573206	AY573223	EU673101
<i>Dothiorella</i> sp.	ColPat-370	<i>J. regia</i> cv. Vina	Antequera, Málaga, Spain	C. Agustí-Brisach & A. Trapero	04/11/2017	MK431833	MK461887	MK522091
	ColPat-371	<i>J. regia</i> cv. Vina	Antequera, Málaga, Spain	C. Agustí-Brisach & A. Trapero	04/11/2017	MK431834	MK461888	MK522092
	ColPat-470*	<i>J. regia</i> cv. Chandler	Antequera, Málaga, Spain	C. Agustí-Brisach & A. Trapero	04/11/2017	MK431839	MK461893	MK522093
<i>Dothiorella vidmadera</i>	CBS 725.79	n/d	n/d	n/d	n/d	KX464130	KX464622	KX464888
<i>Dothiorella viticola</i>	CBS 117009	<i>Vitis vinifera</i> cv. Garnatxa Negra	Spain	n/d	n/d	KF766228	AY905559	EU673104
<i>Lasiodiplodia citricola</i>	CBS 124707 ^T ; IRAN1522C	<i>Citrus</i> sp.	Iran	J. Adbollahzadeh & A. Javadi	n/d	GU945354	GU945340	KU887505
	6I34	<i>J. regia</i>	Stanislaus, CA	T. J. Michailides	10/06/2011	KF778809	KF778999	KF778904
<i>Lasiodiplodia theobromae</i>	CBS 164.96 ^T	Fruit on coral reef coast	New Guinea	A. Aptroot	n/d	AY640255	AY640258	EU673110
<i>Neofusicoccum mediterraneum</i>	CBS 124.13	n/d	U.S.A.	J. J. Taubenhau	n/d	DQ458890	DQ458875	DQ458858
	ColPat-368	<i>J. regia</i> cv. Vina	Antequera, Málaga, Spain	C. Agustí-Brisach & A. Trapero	04/11/2017	MK431831	MK461885	MK522094
	ColPat-369	<i>J. regia</i> cv. Vina	Antequera, Málaga, Spain	C. Agustí-Brisach & A. Trapero	04/11/2017	MK431832	MK461886	MK522095

(Continued on next page)

^x Sequences from GenBank used in the phylogenetic analysis indicated in bold type. T = ex-type isolates; AR and DP = isolates in culture collection of the Systematic Mycology and Microbiology Laboratory, USDA-ARS, Beltsville, MD, U.S.A.; CAA = A. Alves, Universidade de Aveiro, Portugal; CBS = culture collection of the Centraalbureau voor Schimmelcultures, Fungal Biodiversity Centre, Utrecht, The Netherlands; CMW = culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa; ColPat = "Colección Patología", Department of Agronomy, University of Cordoba, Spain; IMI = CABI Bioscience, Egham, U.K.; IRAN = Department of Botany, Iranian Research Institute of Plant Protection, Tehran, Iran; and PD = Plant Protection Service, Wageningen, The Netherlands. Asterisk (*) indicates isolates selected for morphological characterization, study of the effect of temperature on mycelial growth, and pathogenicity tests.

^y Collection date = month/day/year; and n/d = nondetermined.

^z ITS = internal transcribed spacer; EF = translation elongation 1- α ; and TUB = β -tubulin-2 gene regions.

Table 2. (Continued from previous page)

Species	Isolate ^x	Host/cultivar	Origin	Collector	Date ^y	GenBank accession number ^z		
						ITS	EF	TUB
<i>Neofusicoccum parvum</i>	ColPat-556*	<i>J. regia</i> cv. Vina	Badajoz, Spain	C. Agustí-Brisach & A. Trapero	11/03/2017	MK431846	MK461900	MK522096
	ColPat-561	<i>J. regia</i> cv. Chandler	Badajoz, Spain	C. Agustí-Brisach & A. Trapero	11/03/2017	MK431849	MK461903	MK522097
	CBS 121718^T; PD312	<i>Eucalyptus</i> sp.	Greece	P. W. Crous, M. J. Wingfield, & A. J. L. Phillips	n/d	GU251176	GU251308	GU251836
	1H96	<i>J. regia</i>	Selma, Fresno, CA	T. J. Michailides	09/15/2006	KF778811	KF779001	KF778906
	CAA002	<i>Pistacia vera</i> cv. Kerman	U.S.A.	T. J. Michailides	n/d	EU017537	EU017538	KX505925
	Bot-04	<i>Vitis vinifera</i> cv. Pedro Ximénez	Montilla, Córdoba, Andalusia, Spain	C. Agustí-Brisach & A. Trapero	2016	MG745841	MG745817	MG745803
	ColPat-447	<i>J. regia</i> cv. Serr	Benavides, Badajoz, Spain	C. Agustí-Brisach & A. Trapero	07/14/2017	MK431837	MK461891	MK522098
	ColPat-526	<i>J. regia</i> cv. Chandler	Alcalá del Río, Sevilla, Spain	C. Agustí-Brisach & A. Trapero	10/18/2017	MK431841	MK461895	MK522099
	ColPat-528	<i>J. regia</i> cv. Chandler	Alcalá del Río, Sevilla, Spain	C. Agustí-Brisach & A. Trapero	10/18/2017	MK431842	MK461896	MK522100
	ColPat-554*	<i>J. regia</i> cv. Hartley	Badajoz, Spain	C. Agustí-Brisach & A. Trapero	11/03/2017	MK431845	MK461899	MK522101
	ColPat-558	<i>J. regia</i> cv. Serr	Badajoz, Spain	C. Agustí-Brisach & A. Trapero	11/03/2017	MK431848	MK461902	MK522102
<i>Neoscytalidium dimidiatum</i>	ColPat-562	<i>J. regia</i> cv. Chandler	Badajoz, Spain	C. Agustí-Brisach & A. Trapero	11/03/2017	MK431850	MK461904	MK522103
	ColPat-563	<i>J. regia</i> cv. Chandler	Badajoz, Spain	C. Agustí-Brisach & A. Trapero	11/03/2017	MK431851	MK461905	MK522104
	CMW9081^T	<i>Pinus nigra</i>	New Zealand	G. J. Samuels	n/d	AY236943	AY236888	AY236917
	1L83	<i>J. regia</i>	Butte, CA	T. J. Michailides	11/01/2005	KF778854	KF779044	KF778949
	CBS 499.66	<i>Mangifera indica</i>	Mali	n/d	n/d	FM211432	EU144063	FM211167
<i>Diaporthe analyses</i>	CBS 129521^T	<i>Acacia retinodes</i>	Australia	P. W. Crous, I. G. Pascoe, & J. Edwards	n/d	KC343005	KC343731	KC343973
	CBS 495.72^T	<i>Betula alleghaniensis</i>	Ontario, Canada	R. H. Arnold	n/d	FJ889444	GQ250298	KC343975
	CBS 146.46	<i>Alnus</i> sp.	n/d	S. Truter	n/d	KC343008	KC343734	KC343976
	DP0482	<i>Arctium lappa</i>	Austria	W. Jaklitsch	n/d	KJ590736	KJ590776	KJ610891
	CBS 136967	<i>Vaccinium ashei</i>	Chile	A. Schilder	n/d	KJ160562	KJ160594	KJ160518
	CBS 111886^T	<i>Vitis vinifera</i>	Australia	R. W. A. Schepers	n/d	KC343038	KC343764	KC344006
	CBS 454.81	<i>Chamaerops humilis</i>	Greece	H. A. van der Aa	n/d	KC343048	KC343774	KC344016
	CBS 753.70	<i>Spartium junceum</i>	Croatia	J. A. von Arx	n/d	KC343049	KC343775	KC344017
	CBS 719.96	<i>Ficus carica</i>	Bulgaria	E. Ilieva	n/d	KC343050	KC343776	KC344018
	CBS 145.26^T	<i>Carya glabra</i>	Michigan, U.S.A.	L. E. Wehmeyer	n/d	FJ889446	GQ250309	KC344086
	CBS 133813^T	<i>Maytenus ilicifolia</i>	Brazil	R. R. Gomes	n/d	KC343123	KC343849	KC344091
<i>Diaporthe cinerascens</i>	ColPat-445	<i>J. regia</i> cv. Tulare	Benavides, Badajoz, Spain	C. Agustí-Brisach & A. Trapero	07/14/2017	MK447993	MK490932	MK522106
	ColPat-448*	<i>J. regia</i> cv. Serr	Benavides, Badajoz, Spain	C. Agustí-Brisach & A. Trapero	07/14/2017	MK447994	MK490939	MK522107
	ColPat-450	<i>J. regia</i> cv. Vina	Córdoba, Spain	C. Agustí-Brisach & A. Trapero	07/14/2017	MK447996	MK490934	MK522109
	ColPat-451	<i>J. regia</i> cv. Vina	Córdoba, Spain	C. Agustí-Brisach & A. Trapero	07/14/2017	MK447997	MK490935	MK522110
	ColPat-532	<i>J. regia</i> cv. Chandler	Alcalá del Río, Sevilla, Spain	C. Agustí-Brisach & A. Trapero	10/18/2017	MK447998	MK490936	MK522111
	ColPat-551	<i>J. regia</i> cv. Hartley	Badajoz, Spain	C. Agustí-Brisach & A. Trapero	11/03/2017	MK448000	MK490940	MK522112
	ColPat-552	<i>J. regia</i> cv. Hartley	Badajoz, Spain	C. Agustí-Brisach & A. Trapero	11/03/2017	MK448001	MK490938	MK522113
	ColPat-555	<i>J. regia</i> cv. Serr	Badajoz, Spain	C. Agustí-Brisach & A. Trapero	11/03/2017	MK448002	MK490941	MK522114

(Continued on next page)

the data of different loci were combined into single concatenated datasets because no supported nodes were in conflict.

Two separate phylogenetic analyses were conducted to identify Botryosphaeriaceae and *Diaporthe* species. To infer the phylogeny of isolates belonging to Botryosphaeriaceae, the combined alignment of the ITS, EF, and TUB loci was analyzed. In this analysis, a total of 47 taxa were included, of which 23 were the representative isolates of Botryosphaeriaceae used in this study, 23 were GenBank taxa selected based on their high similarity with our query sequences using MegaBLAST, and one was *Neo. dimidiatum* isolate CBS 499.66, which was used as an outgroup (Table 2). In the case of *Diaporthe* isolates, the combined alignment of the TUB and EF loci was conducted ($P = 0.56$). In this analysis, a total of 34 taxa sequences were included, of which 13 were the representative isolates of *Diaporthe* used in this study, 20 were GenBank sequences selected based on their high similarity with our query sequences using MegaBLAST, and one was *Diaporthella corylina* isolate CBS 121124, which was used as an outgroup (Table 2).

In both cases, GenBank sequences were added to the sequences obtained and aligned by Clustal W. Subsequently, maximum parsimony (MP) analyses by the tree-bisection-regrafting algorithm with search level one in which the initial trees were obtained by the random addition of sequences (10 replicates). Adding sequences, alignments, and MP analyses were performed using MEGA version 7.0 software (Kumar et al. 2016). Gaps and missing data were treated as complete deletions. The robustness of the topology was evaluated by 2,000 bootstrap replications (Felsenstein 1985). Measurements for the MP analyses, namely, tree length (TL), consistency index (CI), retention index (RI), homoplasy index (HI), and rescaled consistency index (RC) were calculated for the resulting tree. Additionally, Bayesian inference (BI) analyses were performed by MrBayes version 3.2.6 (Ronquist et al. 2012) using Markov chain Monte Carlo (MCMC) methods to estimate the posterior probability of trees. Models of nucleotide substitution used for each gene partition were also determined by MEGA version 7.0 (Kumar et al. 2016) and included

in the analyses. Two analyses with four MCMC chains each were run simultaneously for 1×10^7 generations, starting from a random tree topology. Trees were sampled every 100 generations, and the “temperature” parameter was set to 0.2. The first 25% of the saved trees was discarded as the burn-in phase of the analysis. Finally, the ITS, EF, and TUB sequences of *Cytospora* spp. isolates were directly BLAST searched in GenBank (<https://www.ncbi.nlm.nih.gov/genbank/>). Sequences derived in this study were lodged at GenBank, and their GenBank accession numbers are shown in Table 2.

Pathogenicity tests. *Fungal isolates.* All inoculations were performed using isolates of Botryosphaeriaceae, *Diaporthe*, and *Cytospora* species (10 isolates in total were selected; Table 2). For inoculum preparation, all the isolates were grown on PDA at 25°C in darkness for 10 days.

Inoculation of detached shoots. In April 2018, 1-year-old detached shoots of walnut cv. Chandler were collected from a 10-year-old experimental orchard belonging to the Andalusian Institute for Research and Formation in Agriculture and Fishery (IFAPA in Spanish), located in Alcalá del Río (Sevilla Province, Andalusia region). Branch segments approximately 25 cm in length were cut and immediately sealed at both ends with Parafilm (Parafilm, Menasha, WI) to reduce desiccation. Subsequently, they were maintained at 4°C until they were processed in the laboratory. Prior to inoculation, the bark surface was cleaned in a water bath with 0.02% Tween 20 for 1 min, surface disinfected for 1 min in 20% sodium hypochlorite solution, washed twice with distilled water, and air dried on sterile filter paper for 30 min. The outer bark was removed from the center of the branch segment using a 7.5-mm-diameter cork borer, and branch segments were placed in humid chambers (plastic containers, 32 × 25 × 8 cm). Subsequently, they were inoculated with a 7.5-mm-diameter mycelium plug obtained from the actively growing margin of the fungal colony, which was deposited in contact with the wounded tissue, and the inoculated point was immediately wrapped with Parafilm. Inoculated shoots were incubated at 27°C and 100% relative humidity for 3 weeks. There were three replicated humid chambers per fungal

Table 2. (Continued from previous page)

Species	Isolate ^x	Host/cultivar	Origin	Collector	Date ^y	GenBank accession number ^z		
						ITS	EF	TUB
<i>Diaporthe phaseolorum</i>	ColPat-559	<i>J. regia</i> cv. Chandler	Badajoz, Spain	C. Agustí-Brisach & A. Trapero	11/03/2017	MK448003	MK490942	MK522115
	ColPat-560	<i>J. regia</i> cv. Chandler	Badajoz, Spain	C. Agustí-Brisach & A. Trapero	11/03/2017	MK448004	MK490943	MK522116
	CBS 123208^T	<i>Foeniculum vulgare</i>	Vora, Portugal	A. J. L. Phillips	n/d	EU814480	GQ250315	KC344072
	CBS 111553^T	<i>F. vulgare</i>	Spain	A. J. L. Phillips	n/d	KC343101	KC343827	KC344069
	6130	<i>J. regia</i>	Stanislaus, CA	T. J. Michailides	10/06/2011	KF778871	KF779061	KF778966
<i>Diaporthe rhusicola</i>	AR4203	<i>Phaseolus vulgaris</i>	U.S.A.	N. F. Gregory	n/d	KJ590738	KJ590739	KJ610893
<i>Diaporthe sp.</i>	ColPat-444*	<i>J. regia</i> cv. Tulare	Benavides, Badajoz, Spain	C. Agustí-Brisach & A. Trapero	07/14/2017	MK447992	MK490931	MK522105
	6115	<i>Prunus dulcis</i>	Kings, CA	T. J. Michailides	09/12/2011	KF778873	KF779063	KF778968
	6131	<i>J. regia</i>	Stanislaus, CA	T. J. Michailides	10/06/2011	KF778874	KF779064	KF778969
	6143	<i>J. regia</i>	Stanislaus, CA	T. J. Michailides	10/06/2011	KF778875	KF779065	KF778970
<i>Diaporthella corylina</i>	ColPat-449	<i>J. regia</i> cv. Serr	Benavides, Badajoz, Spain	C. Agustí-Brisach & A. Trapero	07/27/2017	MK447995	MK490933	MK522108
<i>Phomopsis amygdali</i>	CBS 121124	<i>Corylus</i> sp.	Fuyuan, China	L. N. Vassiljeva	n/d	KC343004	KC343730	KC343972
<i>Cytospora</i> BLAST	ColPat-533*	<i>J. regia</i> cv. Chandler	Alcalá del Río, Sevilla, Spain	C. Agustí-Brisach & A. Trapero	10/18/2017	MK447999	MK490937	MK522117
	CBS 126679^T	<i>P. dulcis</i>	Portugal	E. Diogo	n/d	KC343022	KC343748	KC343990
	CBS 115620	<i>Prunus persica</i>	Georgia, U.S.A.	W. Uddin	n/d	KC343020	KC343746	KC343988
<i>Cytospora</i> sp.	ColPat-465*	<i>J. regia</i> cv. Chandler	Guadix, Granada, Spain	C. Agustí-Brisach & A. Trapero	07/26/2017	MK480242	MK490946	MK546589
	ColPat-633	<i>J. regia</i> cv. Chandler	Guadix, Granada, Spain	C. Agustí-Brisach & A. Trapero	07/19/2018	MK480241	MK490944	MK546587
	ColPat-634	<i>J. regia</i> cv. Chandler	Guadix, Granada, Spain	C. Agustí-Brisach & A. Trapero	07/19/2018	MK480240	MK490945	MK546588

isolate and five shoots per humid chamber. Fifteen additional shoots (three humid chambers, five shoots in each) treated with 7.5-mm-diameter PDA plugs were included as controls. A completely randomized block design was used with the fungal isolate as the independent variable, the shoots as replications, and the humid chambers as blocks. For each treatment (inoculated isolates and control), the lesion length of each shoot was recorded at 3 weeks after

inoculation, and the measures of the five replicated shoots of each block (humid chamber) was averaged. Per humid chamber, one inoculated or noninoculated control shoot was selected for reisolation, which were performed from the margin of the necrotic lesions of inoculated shoots or from the wounded area of control shoots. Reisolation was conducted onto APDA as described above, and the percentage of reisolation was calculated.

Fig. 1. Symptoms of branch dieback and shoot blight of English walnut in southern Spain. **A**, Decayed branch showing necrotic foliage; **B**, typical shoot blight symptoms on young shoots; **C** to **G**, detailed disease progression on shoots and branches showing discoloration on the affected wound (orange arrows); arrows in **E** and **F** point to development of pycnidia on the affected areas of the shoots.

Inoculation of attached shoots under field conditions. One-year-old shoots of walnut cv. Chandler were selected from the same experimental orchard described above. The area of the inoculation was surface disinfested by spraying with 70% ethanol solution, and inoculations were conducted with mycelial plugs as described above. Inoculation was performed on April 19, 2018. Six walnut trees were randomly selected, and two shoots per isolate were inoculated in each tree (one shoot in the north face and the second one in the south face of the tree canopy), with 12 inoculated shoots per isolate and 120 shoots in total. Additionally, 12 shoots (two branches per replicated tree) were treated with 7.5-mm-diameter PDA plugs as a control. A completely randomized block design was used with fungal isolates as the independent variable, the shoots as replications, and the trees as blocks. For each treatment (inoculated isolates and control), the lesion length of each shoot was recorded at 3 months after inoculation (July 19, 2018), and the measures of the two replicated shoots of each block (tree) were averaged. Three inoculated shoots per isolate and three noninoculated control shoots were selected for reisolations, which were performed as described above. Additionally, reisolations from asymptomatic tissues 2 cm beyond the margin of the lesion or the wounded area from both inoculated and control shoots were also performed. All the reisolations were conducted onto APDA as described above, and the percentage of reisolation in each tissue was calculated.

Inoculation of detached walnut fruits. Green fruits of walnut cv. Chandler were collected from the same experimental orchard described above. Fruits were surface disinfested following the same protocol described for detached shoots. Disinfested walnut fruits were placed in humid chambers, and a wound of 5-mm diameter × 4-mm depth was made on the center of the hull of each fruit using a cork borer. Inoculations were conducted by placing a 5-mm-diameter mycelial plug inside the hole. There were three replicated humid chambers per treatment, 10 fruits per humid chamber, 30 fruits per treatment, and 300 fruits in total. Additionally, 30 fruits (three humid chambers, 10 fruits in each) treated with a 5-mm-diameter PDA plug were included as controls. A completely randomized block design was used with fungal isolates as the independent variable, the fruits as replications, and the humid chambers as blocks. Disease severity was evaluated every 3 days until most of the fruits inoculated with the most aggressive isolate had 90 to 100% of their surface affected. The major and minor diameters of each lesion were recorded and averaged for each evaluation, and the percentage of the affected fruit surface of each lesion in each treatment and evaluation was calculated. The relative area under the disease progress curve (RAUDPC) was obtained by trapezoidal integration (Campbell and Madden 1990) for each inoculated or control fruit, and the RAUDPC values of the 10 fruits of each block (humid chamber)

were averaged. Fungal reisolation was conducted from the margin of the necrotic lesions as described above. Additionally, reisolations from the affected or asymptomatic tissues around the peduncle were also performed from both inoculated and control fruits. All the reisolations were conducted onto APDA as described above, and the percentage of reisolation in each tissue was calculated.

Data analysis. The dependent variables lesion length and RAUDPC were subjected to ANOVA to determine the differences in aggressiveness between fungal isolates on infected shoots and green fruits of walnut, respectively. In all cases, data were tested for normality and homogeneity of variances and logarithmically transformed when necessary. In all cases, control treatments were excluded from the statistical analysis because no lesions were observed in noninoculated control shoots or green fruits, and treatment means were compared according to Tukey's HSD test at $\alpha = 0.05$ (Steel and Torrie 1985). The Pearson correlation coefficient (r) between the lesion length and diameter of the shoots was calculated using the average of the two measures for the fungal isolates evaluated ($n = 10$). Additionally, Pearson's linear correlation was also conducted to compare infections between inoculated detached and attached shoots and detached shoots and fruits using the average lesion length or RAUDPC values for infected shoots and fruits, respectively, for all isolates evaluated ($n = 10$). All data of this study were analyzed using Statistix 10 software (Analytical Software 2013).

Results

Field surveys and fungal isolation. Typical dieback symptoms, including twig and leaf blight, were observed in association with shoot blight and wood canker formation with internal discoloration (Fig. 1). Fungal fruiting structures were usually found on affected tissues (Fig. 1E and F). From the samples collected during the field surveys, 470 out of 630 attempts of isolation from symptomatic tissues yielded a fungal culture (74.6%). The isolation of *Botryosphaeria* fungi was the most consistent with 53.0% positive inoculation points, followed by *Diaporthe* fungi with 38.0% positive inoculation points. These two groups of fungi were recovered alone or simultaneously from seven out of the 10 surveyed orchards. The cooccurrence of *Botryosphaeria* and *Diaporthe* fungi was observed in five out of 10 surveyed orchards. *Cytospora* sp. was isolated alone from a single orchard (Guadix, Granada) with a low frequency of isolation (9.0%).

Fungal colony, conidia, and pycnidia morphology. According to their appearance in culture (texture, density, color, zonation, margin, and growth rate), the isolates were first assigned to three main fungal groups. The first group comprised colonies associated with typical characteristics of *Botryosphaeria*. In general, all isolates of this first group showed floccose to felted fast-growing aerial

Table 3. Morphological characteristics of the representative fungal isolates collected from English walnut showing branch dieback and shoot blight in southern Spain

Family/fungal species	Isolate ^z	Mycelia				
		Obverse			Reverse	
		Color	Zonation	Margin	Color	Zonation
Botryosphaeriaceae						
<i>Botryosphaeria dothidea</i>	ColPat-443	White gray	No	Irregular	Dark to olive green	No
<i>Diplodia seriata</i>	ColPat-557	Light gray-brown	No	Irregular	Light to dark gray	No
<i>Dothiorella sarmentorum</i>	ColPat-471	Dark to olive brown	Yes	Irregular	Dark gray to olive green	Yes
<i>Dothiorella</i> sp.	ColPat-470	Dark gray to olive green	No	Regular	Dark gray to olive green	No
<i>Neofusicoccum mediterraneum</i>	ColPat-556	Light gray to olive green	No	Irregular	Olive green	No
<i>Neofusicoccum parvum</i>	ColPat-554	White to light gray	No	Regular	White to light gray	No
Diaporthaceae						
<i>Diaporthe neotheicola</i>	ColPat-448	White beige	No	Irregular	White beige	No
<i>Diaporthe rhusicola</i>	ColPat-444	Light brown to white beige	No	Irregular	Light brown to white beige	Yes
<i>Phomopsis amygdali</i>	ColPat-533	Light brown to white beige	No	Irregular	Light to dark brown	No
Valsaceae						
<i>Cytospora</i> sp.	ColPat-465	White beige to blue-olive gray	No	Irregular	Dark gray to olive green	No

^z Single conidial cultures were grown on PDA for 7 days at 25 ± 2°C in darkness, and colony color was determined by means of a color scale (Kornerup and Wanscher 1963).

mycelium with colors varying from white-gray to dark gray or olive green on PDA (Table 3; Fig. 2A to F). With age (from 10 to 14 days of incubation), all Botryosphaeriaceae isolates developed single or grouped, dark-brown to black-dark, ostiolate, globose, erumpent pycnidia, varying in diameter from 286.3 to 769.4 μm , on the surface of pistachio leaves on LA. When conidia from pycnidia were observed, Botryosphaeriaceae isolates were classified at the genus level resulting in the following four subgroups: (i) *Botryosphaeria*: aseptate, fusiform elliptical, hyaline conidia; (ii) *Diplodia*: aseptate, rounded, brown conidia; (iii) *Dothiorella*: (zero-) to one-septate, ovoid, hyaline becoming dark brown conidia; and (iv) *Neofusicoccum*: aseptate, fusoid-ellipsoidal (fusiform), hyaline conidia (Table 4). The second group was classified as *Diaporthe* because the colonies showed white, cottony, slow-growing, and raised mycelium developing prominent growth rings with irregular margins that became dark brown with age (Table 3; Fig. 2G to I). All isolates of the second group developed pycnidia over time on LA as well as on PDA, showing a characteristic white-beige-colored conidial mass on the pycnidia. Fruiting bodies were dark, ostiolate, globose, erumpent, and velvety and varied in diameter (216.1 to 354.9 μm). Two types of hyaline conidia were observed: alpha conidia (α): small, oblong, slightly fusiform, and ligulated conidia; and beta conidia (β): filiform, smooth, slightly curved conidia (Table 4). Finally, a third group was composed of only one type of colony that developed white-beige to blue-olive-gray, flat, uniform mycelium with irregular colony margins (Table 3; Fig. 2J). Pycnidia were formed on LA after 14 days of incubation, and they were dark, erumpent, ostiolate, tuberculate, globous, velvet, and 485.2 μm in diameter. Conidia were aseptate, elongate-allantoid, smooth-walled and hyaline (Table 4). This group of colonies was identified morphologically as *Cytospora* sp.

Effect of temperature on mycelial growth. All isolates used in this experiment were able to grow on PDA from 10 to 30°C, showing little or sometimes a lack of mycelial growth development at both extreme temperatures tested (5 and 35°C). In general, different growth patterns were observed between the three fungal groups, and significant differences in optimum growth temperature and MGR ($P < 0.0001$ in any cases) were observed between isolates. Optimum growth temperatures ranged from 19.5 to 27.7°C, with the *Cytospora* sp. isolate ColPat-465 having the lowest growth temperature and the *B. dothidea* isolate ColPat-443 having the highest, and MGR ranged from 17.0 to 2.4 mm/day, with the *N. parvum* isolate ColPat-554 having the lowest MGR and the *Dia. neotheicola* isolate ColPat-448 having the highest. Among Botryosphaeriaceae isolates, the *B. dothidea* isolate ColPat-443 had the highest optimum growth temperature (27.7°C), followed by the *D. seriata* isolate ColPat-557 (26.6°C). Isolates belonging to the *Dothiorella* genus had the lowest optimum growth temperatures among the Botryosphaeriaceae isolates (19.6 and 22.0°C for the *Dot. sarmentorum* isolate ColPat-471 and *Dothiorella* sp. ColPat-470, respectively). The *N. parvum* isolate ColPat-554 showed the highest MGR value (17.0 mm/day), whereas *N. mediterraneum* showed the lowest MGR value (5.1 mm/day) among Botryosphaeriaceae isolates. Concerning *Diaporthe* species, the optimum growth temperature ranged from 23.2 to 26.6°C, with the *Dia. rhusicola* isolate ColPat-444 having the lowest growth temperature and the *Dia. neotheicola* isolate ColPat-448 having the highest; MGR ranged from 2.4 to 6.4 mm/day, with the *Dia. neotheicola* isolate ColPat-448 having the highest MGR and the *Ph. amygdali* isolate ColPat-533 having the lowest. Finally, the *Cytospora* sp. isolate ColPat-465 showed the lowest optimum growth temperature (19.5°C) of the entire experimental group with an MGR of 4.3 mm/day (Table 5; Fig. 3; for interpretation of the results of this experiment with respect to the species name of each isolate, the reader is referred to the following section of this article).

Molecular characterization. Botryosphaeriaceae isolates were inferred by analyzing the combined alignment of the ITS, EF, and TUB sequences. This analysis contained 47 taxa, including the out-group *Neo. dimidiatum* isolate CBS 499.66. The gene boundaries were as follows: ITS, 1 to 511; EF, 512 to 779; and TUB, 780 to 1,122. A total of 1,122 characters, including gaps, were processed,

of which 305 characters were parsimony informative and 788 were conserved sites. All positions containing gaps and missing data were eliminated when the MP analysis was performed. The MP analysis showed eight most parsimonious trees (TL = 327; CI = 0.757;

Fig. 2. Two-week-old colonies of the different fungal species isolated from English walnut in southern Spain growing on PDA at 25°C in darkness. Botryosphaeriaceae isolates: **A**, *Botryosphaeria dothidea* isolate ColPat-443; **B**, *Diplodia seriata* isolate ColPat-557; **C**, *Dothiorella sarmentorum* isolate ColPat-471; **D**, *Dothiorella* sp. isolate ColPat-470; **E**, *Neofusicoccum mediterraneum* isolate ColPat-556; and **F**, *Neofusicoccum parvum* isolate ColPat-554. *Diaporthe* isolates: **G**, *Diaporthe neotheicola* isolate ColPat-448; **H**, *Diaporthe rhusicola* isolate ColPat-444; and **I**, *Phomopsis amygdali* isolate ColPat-533. **J**, *Cytospora* sp. isolate ColPat-465.

RI = 0.962; HI = 0.243; RC = 0.728), of which one is shown in Figure 4. For BI analysis, the K2+G and T92+G models were used for the ITS region and for the EF and TUB genes, respectively. The tree obtained by BI analysis confirmed the topology obtained with MP. In this phylogenetic tree, the combined sequences of the Botryosphaeriaceae isolates clustered in six well-supported clades: (i) four isolates (ColPat-443, ColPat-446, ColPat-530, and ColPat-553) clustered together with reference GenBank sequences of *B. dothidea* (bootstrap support [BS; %]/Bayesian posterior probability [PP]: 100/1.00); (ii) three isolates (ColPat-366, ColPat-367, and ColPat-557) clustered together with reference GenBank sequences of *D. seriata* (BS/PP: 99/1.00); (iii) three isolates (ColPat-370, ColPat-371, and ColPat-470) clustered together with reference GenBank sequences of *Dot. omni-vora* and *Diplodia juglandis* (BS/PP: 97/1.00), considering our isolates as *Dothiorella* sp.; (iv) two isolates (ColPat-469 and ColPat-471) clustered together with reference GenBank sequences of *Dot. sarmentorum* (BS/PP: 93/0.97); (v) four isolates (ColPat-368, ColPat-369, ColPat-556, and ColPat-561) clustered together with reference GenBank sequences of *N. mediterraneum* (BS/PP: 100/1.00); and (vi)

seven isolates (ColPat-447, ColPat-526, ColPat-528, ColPat-554, ColPat-558, ColPat-562, and ColPat-563) clustered together with reference GenBank sequences of *N. parvum* (BS/PP: 100/1.00).

With respect to the *Diaporthe* isolates, they were inferred by analyzing a combined alignment of the TUB and EF sequences. This analysis contained 34 taxa, including the outgroup *Diaporthella corylina* isolate CBS 121124. The gene boundaries were as follows: TUB, 1 to 481; and EF, 472 to 819. A total of 819 characters including gaps were processed, of which 329 characters were parsimony informative and 275 were conserved sites. All positions containing gaps and missing data were eliminated when the MP analysis was performed. The MP analysis showed four most parsimonious trees (TL = 724; CI = 0.722; RI = 0.805; HI = 0.278; RC = 0.581), of which one is shown in Figure 5. For BI analysis, the HKY+G model was used for both EF and TUB genes. The tree obtained by BI analysis confirmed the topology obtained with MP. In this phylogenetic tree, the combined sequences of the *Diaporthe* isolates clustered in three well-supported clades: (i) one isolate (ColPat-533) clustered together with reference GenBank sequences of *Ph. amygdali* (BS/PP: 100/1.00);

Table 4. Morphological characteristics of conidia of the representative fungal isolates collected from English walnut showing branch dieback and shoot blight in southern Spain

Family/fungal species	Isolate	Conidia				Pycnidia	
		Length × width (μm) ^x		Length/width ^y	Morphology	Color	Diameter (μm) ^z
Botryosphaeriaceae							
<i>Botryosphaeria dothidea</i>	ColPat-443	(20.5–) 24.4 (–29.2) × (3.0–) 5.2 (–7.2)	4.9 ± 0.22	Aseptate, fusiform elliptical with a subobtuse apex and a truncate base bearing a minute basal frill.	Hyaline	556.1 ± 26.2	
<i>Diplodia seriata</i>	ColPat-557	(18.0–) 21.5 (–24.6) × (7.4–) 9.9 (–11.6)	2.2 ± 0.05	Aseptate, inner surface of wall finely roughened, apex broadly rounded, base truncate.	Brown	347.1 ± 13.0	
<i>Dothiorella sarmentorum</i>	ColPat-471	(16.9–) 19.1 (–21.2) × (7.1–) 9.0 (–11.3)	2.2 ± 0.04	(Zero–) to one-septate, slightly constricted at the septum, ovoid with a broadly rounded apex and truncate base, brown walled.	Hyaline, becoming pigmented brown	554.9 ± 21.0	
<i>Dothiorella</i> sp.	ColPat-470	(13.7–) 17.3 (–21.9) × (5.3–) 7.5 (–10.3)	2.4 ± 0.15	One-septate, ovoid with a broadly rounded apex and truncate base, brown walled.	Hyaline, becoming dark brown	346.3 ± 22.2	
<i>Neofusicoccum mediterraneum</i>	ColPat-556	(13.7–) 18.4 (–21.1) × (4.3–) 5.8 (–7.1)	3.23 ± 0.08	Aseptate, smooth, thin-walled, fusoid-ellipsoidal, widest in the middle-upper third, apex subobtuse, subtruncate base.	Hyaline	286.3 ± 17.9	
<i>Neofusicoccum parvum</i>	ColPat-554	(12.3–) 16.9 (–23.5) × (4.8–) 5.9 (–7.2)	2.9 ± 0.02	Aseptate, ellipsoid with subobtuse apex and truncate base, smooth thin-walled, becoming darker before germination.	Hyaline	769.4 ± 25.8	
Diaporthaceae							
<i>Diaporthe neotheicola</i>	ColPat-448	(4.8–) 6.6 (–8.7) × (1.1–) 1.9 (–2.9)	3.7 ± 0.15	α-Conidia: Aseptate, subcylindrical to fusoid-ellipsoidal, widest in middle.	Hyaline	354.9 ± 15.7	
<i>Diaporthe rhusicola</i>	ColPat-444	(5.6–) 6.7 (–8.1) × (1.1–) 2.2 (–3.0)	3.2 ± 0.15	α-Conidia: Aseptate, subcylindrical to fusoid-ellipsoidal, widest in middle.	Hyaline	287.5 ± 10.4	
<i>Phomopsis amygdali</i>	ColPat-533	(3.7–) 5.6 (–7.3) × (1.1–) 1.8 (–2.9)	3.3 ± 0.15	α-Conidia: Aseptate, subcylindrical to fusoid-ellipsoidal, widest in middle.	Hyaline	216.1 ± 13.7	
Valsaceae							
<i>Cytospora</i> sp.	ColPat-465	(3.1–) 3.9 (–4.6) × (0.7–) 1.1 (–1.6)	3.7 ± 0.16	Aseptate, elongate-allantoid, smooth-walled.	Hyaline	485.2 ± 19.8	

^x Mean and range values: length × width (μm). The extremes of the conidial measurements are shown inside parenthesis. Values represent the mean of 30 conidia.

^y Conidia length/width ratio. Values represent the mean of 30 conidia ± standard error of the mean.

^z Values represent the mean of the diameter (μm) of 30 pycnidia ± standard error of the mean.

(ii) 10 isolates (ColPat-445, ColPat-448, ColPat-450, ColPat-451, ColPat-532, ColPat-551, ColPat-552, ColPat-555, ColPat-559, and ColPat-560) clustered together with reference GenBank sequences of *Dia. neotheicola* (BS/PP: 99/1.00); and (iii) one isolate (ColPat-444) clustered together with reference GenBank sequences of *Dia. rhusicola* (BS/PP: 99/1.00). Additionally, one of our isolates (ColPat-449) clustered into this later cluster close to reference GenBank sequences of *Dia. chamaeropsis* and *Dia. rhusicola* (BS/PP: 88/0.99), and we considered this isolate to be *Diaporthe* sp.

Finally, the ITS, EF, and TUB sequences of the *Cytospora* spp. isolates ColPat-465, ColPat-633, and ColPat-634 were identified by performing a GenBank BLAST search. The ITS, EF, and TUB sequences of the isolates ColPat-465, ColPat-633, and ColPat-634 were incongruent when they were BLAST searched in GenBank. The BLAST searches showed a broad diversity of *Cytospora* species such as *Cytospora abietis*, *C. carbonacea*, *C. cotini*, *C. rhodophila*, *C. prunicola*, *C. ribis*, or *Cytospora* sp. with a similar percentage of identity (from 95 to 99%; data not shown).

Pathogenicity tests. *Inoculation of detached shoots.* All isolates tested were able to induce lesions and internal wood discoloration on the inoculated walnut shoots at 3 weeks after inoculation in laboratory conditions (Fig. 6A). Significant differences in aggressiveness ($P < 0.0001$) were observed within species, with the mean lesion length ranging from 3.6 to 134.3 mm, with the *Cytospora* sp. isolate ColPat-465 inducing the shortest lesion length and the *N. parvum* isolate ColPat-554 inducing the largest. The *N. parvum* isolate ColPat-554 was the most aggressive (lesion length = 134.3 mm), followed by the *B. dothidea* isolate ColPat-443 (lesion length = 130.7 mm) and the *N. mediterraneum* isolate ColPat-556 (lesion length = 112.5 mm). The *Dot. sarmentorum* isolate ColPat-471 was the least aggressive within Botryosphaeriaceae isolates (lesion length = 54.6 mm). With respect to *Diaporthe* fungi, the *Dia. neotheicola* isolate ColPat-448 showed the largest lesions (lesion length = 23.3 mm), followed by the *Ph. amygdali* isolate ColPat-533 (lesion length = 17.6 mm). Finally, the *Cytospora* sp. isolate ColPat-465 was the least aggressive among all the species in the entire experiment (lesion length = 3.6 mm) (Fig. 7A). All fungal species were consistently reisolated from the margin of the necrotic lesion, with a percentage of reisolation ranging from 81.0 to 100%. No positive reisolations were obtained from control shoots (Table 6). A significant negative linear correlation was observed between lesion length (mm) and diameter (mm) ($r = -0.6747$; $P = 0.0323$).

Inoculation of attached shoots under field conditions. All isolates tested were able to induce lesions and internal wood discoloration on the inoculated walnut shoots under field conditions at 3 months after

inoculation (Fig. 6B). Occasionally, serious attacks of branch die-back occurring in dry and dead branches were observed in shoots inoculated with mycelial plugs of the *N. parvum* isolate ColPat-554 (67% of dead shoots), the *D. seriata* isolate ColPat-557 (25% of dead shoots), and the *N. mediterraneum* isolate ColPat-556 (13% of dead shoots) (Fig. 6C). Significant differences ($P < 0.0001$) in aggressiveness were observed within species, with the mean lesion length ranging from 5.1 to 90.5 mm, with the *Cytospora* sp. isolate ColPat-465 inducing the shortest lesion length and the *N. parvum* isolate ColPat-554 inducing the longest. The *N. parvum* isolate ColPat-554 was also the most aggressive (lesion length = 90.5 mm), followed by the *N. mediterraneum* isolate ColPat-556 (lesion length = 28.0 mm). The *Ph. amygdali* isolate ColPat-533 showed similar aggressiveness (lesion length = 13.0 mm) to that observed for the Botryosphaeriaceae species *D. seriata* isolate ColPat-557 (lesion length = 12.5 mm) and *B. dothidea* isolate ColPat-443 (lesion length = 10.9 mm). Finally, the *Cytospora* sp. isolate ColPat-465 was again the least aggressive among all the species in the entire experiment (lesion length = 5.1 mm) (Fig. 7B). Botryosphaeriaceae were the most consistent fungi reisolated from the inoculated shoots under field conditions, with *N. parvum* showing the highest percentage of reisolation (95.2%). *Diaporthe* species were reisolated at a lower percentage (23.8 to 42.9%) than Botryosphaeriaceae fungi (54.8 to 95.2%). *Cytospora* sp. showed the lowest percentage of reisolation (9.5%) among all species in the entire experiment. When reisolations were performed at 2 cm beyond the margin of the necrotic lesions, only *N. parvum* was reisolated at a low percentage (2.4%). No positive reisolations were obtained from any evaluated tissue from control shoots (Table 6). Linear correlation analyses showed that there was no significant correlation ($r = -0.2845$; $P = 0.4256$) between lesion length (mm) and branch diameter (mm). However, a highly significant positive linear correlation ($r = 0.8144$; $P = 0.0041$) was observed between lesion length (mm) data from infected shoots evaluated in both laboratory and field conditions for the 10 fungal isolates tested.

Inoculation of detached walnut fruit. Dark-brown circular necrotic lesions varying in diameter among isolates tested were observed in the epicarp of the inoculated fruit at 3 weeks after inoculation in laboratory conditions. Internal discoloration and consistent fruit rot were observed in most of the inoculated fruit (Fig. 6D). In general, the Botryosphaeriaceae isolates were more aggressive with respect to the *Diaporthe* and *Cytospora* fungi. Significant differences in RAUDPC were observed within species ($P < 0.0001$). The *N. parvum* isolate ColPat-554 was the most aggressive (RAUDPC = 98.8%), followed by the *B. dothidea* isolate ColPat-443 (RAUDPC = 65.3%) and the *N. mediterraneum* isolate ColPat-556 (RAUDPC = 56.7%). In

Table 5. Effect of temperature on mycelial growth of the representative fungal isolates selected in this study grown on PDA at 5, 10, 15, 20, 25, 30, and 35°C in darkness from 3 to 7 days^v

Family/fungal species	Isolate	Analytics Beta model ^w			Temperature (°C) ^{x,y}			MGR (mm/day) ^{z,x}
		R ²	a	b	Optimum	Minimum	Maximum	
Botryosphaeriaceae								
<i>Botryosphaeria dothidea</i>	ColPat-443	0.9888	3.68	1.84	27.7 a	10.0	36.5	15.4 b
<i>Diplodia seriata</i>	ColPat-557	0.9737	3.60	1.41	26.6 b	4.0	35.5	13.4 c
<i>Dothiorella sarmentorum</i>	ColPat-471	0.9561	2.25	2.48	19.6 e	4.5	36.5	5.5 ef
<i>Dothiorella</i> sp.	ColPat-470	0.9112	1.21	0.70	22.0 d	9.0	30.5	9.0 d
<i>Neofusicoccum mediterraneum</i>	ColPat-556	0.9454	1.47	2.43	23.7 c	5.0	35.0	5.1 fg
<i>Neofusicoccum parvum</i>	ColPat-554	0.9924	3.37	1.59	26.0 b	5.0	36.0	17.0 a
Diaporthaceae								
<i>Diaporthe neotheicola</i>	ColPat-448	0.9435	4.66	2.40	26.6 b	10.0	35.0	2.4 h
<i>Diaporthe rhusicola</i>	ColPat-444	0.8579	3.79	2.40	23.2 c	4.5	35.0	4.2 g
<i>Phomopsis amygdali</i>	ColPat-533	0.9942	4.57	1.32	26.3 b	5.0	32.5	6.4 b
Valsaceae								
<i>Cytospora</i> sp.	ColPat-465	0.9878	1.24	1.42	19.5 e	10	30.5	4.3 fg

^v Data represents the average of four replicated Petri dishes per isolate and temperature combination.

^w Analytis Beta model, where R² = coefficient of determination, and a and b = coefficients of regression.

^x For each isolate, temperature average growth rates were adjusted to a regression curve to estimate the optimum growth temperature.

^y Means in a column followed by the same letter do not differ significantly according to Tukey's HSD test at $P = 0.05$ (Steel and Torrie 1985).

^z MGR = maximum growth rate (mm/day) obtained by the Analytics Beta model at the optimum growth temperature.

contrast, the remaining species showed similar effects on fruit infection, with RAUDPC values ranging from 34.0 to 39.8%, with the *Cytospora* sp. isolate ColPat-465 having the lowest RAUDPC value and the *Ph. amygdali* isolate ColPat-533 having the highest (Fig. 8). Most of the fungal species were consistently reisolated from the margin of the necrotic lesion (93.0 to 100%), except for *Dot. sarmentorum* and *Cytospora* sp. which showed 50% reisolation. When reisolations were

performed from the peduncle of the inoculated fruit, only some Botryosphaeriaceae species were reisolated (50.0 to 93.0%), with *N. parvum* being the most consistently reisolated species. No positive reisolations were obtained from any evaluated tissue from control fruit (Table 6). A significant positive linear correlation was observed between RAUDPC (%) and lesion length (mm) data from the detached fruit and shoots inoculated in laboratory conditions ($r = 0.8424$;

Fig. 3. Effect of temperature on mycelial growth rate (mm/day) of the 10 representative isolates selected in this study grown on PDA at 5, 10, 15, 20, 25, 30, and 35°C in darkness for 3 to 7 days. For each isolate, average growth rates over temperature were adjusted to a nonlinear regression curve using the Analytis Beta model. Data points are the means of two independent experiments of four replicated Petri dishes each. Vertical bars are the standard error of the means.

$P = 0.0022$) and from the detached fruit and shoots inoculated under field conditions ($r = 0.9780$; $P < 0.0001$).

Discussion

A total of 10 commercial English walnut orchards showing branch dieback and shoot blight symptoms were surveyed in this study across southern Spain. The symptoms observed included twig and leaf blight associated with shoot blight and wood canker formation with internal discoloration and fungal fruiting structures embedded in the bark of the affected tissues (Fig. 1). Owing to the growing concern for nut growers in this area because the incidence of the disease has increased in the last few years, this study aimed to elucidate the etiology of the disease to provide a consistent diagnosis. This work is the first report to identify the fungal species causing this complex disease of English walnut in Spain and Europe.

All isolates recovered from affected wood tissues were grouped into three fungal groups (Botryosphaeriaceae, *Cytospora*, and *Diaporthe*) according to their morphological characteristics with respect to those described for each family or genus (Dissanayake et al. 2016; Gomes et al. 2013; Norphanphoun et al. 2017; Phillips et al. 2008, 2013, 2018). Botryosphaeriaceae isolates were the most consistently isolated fungi, followed by *Diaporthe* isolates. These two groups of fungi were recovered from seven out of the 10 surveyed orchards. They cooccurred in five orchards showing coinfections in the affected branches. The distribution of the populations of Botryosphaeriaceae and *Diaporthe* isolates in the English walnut plantations in southern Spain seemed to be balanced for the two groups, even though Botryosphaeriaceae isolates were always isolated more consistently than *Diaporthe* isolates. These results differ

from those observed in California, where only 20% of the processed samples showed the cooccurrence of both Botryosphaeriaceae and *Diaporthe* fungi (Agustí-Brisach et al. 2019b; T. J. Michailides, unpublished data). In addition, *Cytospora* was recovered alone from a single orchard in our study, with minor relevance with respect to the other two fungal groups. Recently, this genus has also been associated with walnut canker disease in China (Fan et al. 2015; Zhao et al. 2018). Considering that *Cytospora* fungi caused the lowest incidence of disease, our results are in accordance with those obtained by Zhao et al. (2018), who observed this fungus only in a range from 5 to 10% of severely affected natural walnut forests.

The experiment evaluating the effect of temperature on mycelial growth showed a broad range of variation in the optimum growth temperature (19.5 to 27.7°C) between isolates. In general, Botryosphaeriaceae and *Diaporthe* isolates had high optimum growth temperatures of approximately 26 to 27°C. Our results are in agreement with those obtained by several authors who showed similar ranges of temperature for these two groups of fungi in California and Spain. This information confirms that Botryosphaeriaceae and *Diaporthe* fungi are well adapted to the warm temperatures that occur in these locations, where canker diseases have been described as important diseases of woody crops (Agustí-Brisach et al. 2019a, 2019b; Michailides and Hasey 2010; Moral et al. 2010, 2017, 2019). Moreover, *Cytospora* showed the lowest optimum growth temperature (19.5°C) of all isolated in the entire experiment. This result is also in agreement with such obtained by Lawrence et al. (2017), who characterized *Cytospora* isolates from grapevines in North America. These authors observed that most of the *Cytospora* isolates showed slightly higher growth at 20 than at 25°C.

Fig. 4. One of eight most parsimonious trees (tree length = 327; consistency index = 0.757; retention index = 0.962; homoplasy index = 0.243; rescaled consistency index = 0.728) obtained from a heuristic search of the combined internal transcribed spacer, elongation factor 1- α , and β -tubulin sequence alignment of fungal species belonging to Botryosphaeriaceae. Bootstrap support values (maximum parsimony [MP], >70%) and Bayesian posterior probabilities (PP, >0.9) are shown at the nodes (MP/PP). The asterisk (*) indicates full support (100/1.00). *Neoscytalidium dimidiatum* isolate CBS 499.66 was used as the outgroup.

Fig. 5. One of four most parsimonious trees (tree length = 724; consistency index = 0.722; retention index = 0.805; homoplasy index = 0.278; rescaled consistency index = 0.581) obtained from a heuristic search of the combined β -tubulin and elongation factor 1- α sequence alignment of *Diaporthe* fungi. Bootstrap support values (maximum parsimony [MP], >70%) and Bayesian posterior probabilities (PP, >0.9) are shown at the nodes (MP/PP). The asterisk (*) indicates full support (100/1.00). *Diaphorthea corylina* isolate CBS 121124 was used as the outgroup.

Although the phenotypic characteristics were useful for distinguishing our isolates between families and genera, sequencing and phylogenetic analysis were required to identify them at the species level. Based on previous studies that resolved the taxonomy of Botryosphaeriaceae (Chen et al. 2014; Dissanayake

et al. 2016; Phillips et al. 2013, 2018), *Cytospora* (Norphanphoun et al. 2017), and *Diaporthe* (Chen et al. 2014; Gomes et al. 2013; Udayanga et al. 2014), sequences of the ITS, EF, and TUB gene regions were combined to identify our fungal isolates. Note that in the case of *Diaporthe* spp., the ITS region was not included

Fig. 6. A, Internal discoloration on detached shoots, and **D**, external and internal lesions on green fruit of English walnut cv. Chandler at 3 weeks after inoculation with mycelial plugs of PDA (0 = noninoculated control shoot) and of the 10 representative isolates selected for pathogenicity: 1 = *Neofusicoccum parvum* isolate ColPat-554; 2 = *Botryosphaeria dothidea* isolate ColPat-443; 3 = *N. mediterraneum* isolate ColPat-556; 4 = *Diplodia seriata* isolate ColPat-557; 5 = *Dot. sarmentorum* isolate ColPat-471; 6 = *Dothiorella* sp. isolate ColPat-470; 7 = *Diaporthe neotheicola* isolate ColPat-448; 8 = *Phomopsis amygdali* isolate ColPat-533; 9 = *Diaporthe rhusicola* isolate ColPat-444; and 10 = *Cytospora* sp. isolate ColPat-465. **B** and **C**, internal discoloration and branch dieback, respectively, on shoots of English walnut cv. Chandler at 3 months after inoculation with mycelial plugs of the *N. parvum* isolate ColPat-554 under field conditions.

in the alignment and phylogenetic analysis because previous studies on taxonomy into the genus *Diaporthe* showed a discordance of ITS versus other gene trees and a lack of informative morphological characters creating taxonomic ambiguities within this genus (Udayanga et al. 2014).

Fig. 7. Disease severity (lesion length, mm) on **A**, detached (laboratory conditions) and **B**, attached (field conditions) shoots of English walnut cv. Chandler at 3 weeks and 4 months, respectively, after inoculation with mycelial plugs of representative fungal isolates belonging to the Botryosphaeriaceae (dark gray columns) and the *Diaporthe* (light gray columns) and *Cytospora* (white column) genera. For each isolate, columns represent the mean of six humidity chambers with five replicate shoots each in the artificial inoculations (A) and the mean of six walnut trees with two inoculated shoots in each in the field experiments (B). In each graph, columns with common letters do not differ significantly according to Tukey's HSD test ($\alpha = 0.05$). Vertical lines on the columns are the standard error of the mean.

The following species were identified within the Botryosphaeriaceae group: *B. dothidea*, *D. seriata*, *Dot. sarmentorum*, *Dothiorella* sp., *N. mediterraneum*, and *N. parvum*. All of these species have been reported previously to cause branch dieback of walnut around the world (Chen et al. 2014; Li et al. 2015; Michailides and Hasey 2010), with the exception of *Dot. sarmentorum*, which we reported to be associated with this walnut disease for the first time. Concerning the *Diaporthe* group, most of our *Diaporthe* isolates were identified as *Dia. neotheicola*, making this species the most prevalent species in this group. The remaining isolates of this group were identified as *Diaporthe* sp. (ColPat-449), *Dia. rhusicola* (ColPat-444), and *Ph. amygdali* (ColPat-533). Note that this latter has been traditionally described as the primary causal agent of cankers and wood decay in almond (Diogo et al. 2010; Gramaje et al. 2012; Rhouma et al. 2008). However, the detection of *Ph. amygdali* as a causal agent of branch dieback in the walnut in this study confirms that this fungal species is not restricted to *Prunus* spp. (Gramaje et al. 2012). Indeed, *Ph. amygdali* has also been isolated from other woody plants, such as grapevines in South Africa (Mostert et al. 2001) or *Pieris japonica* in the United States (Bienapfl and Balci 2013). Additionally, *Dia. neotheicola* (= *Dia. foeniculina*) has also been isolated from *Foeniculum vulgare* in Spain (Guarnaccia et al. 2018).

In both Botryosphaeriaceae and *Diaporthe* analyses, there was an isolate in each that was only identified at the genus level. The sequences of the isolates ColPat-370, ColPat-371, and ColPat-470 did not match any specific GenBank reference sequences of Botryosphaeriaceae with a high percentage of similarity ($\leq 96\%$) when they were BLAST searched in GenBank. When the combined alignment was conducted, these isolates were grouped together inside the *Dothiorella* sp. cluster, close to the reference sequences of *D. juglandis* CBS 188.87 (BS/PP: 97/1.0) and *Dot. omnivora* CBS 392.80 (BS/PP: 97/1.0). Owing to the unresolved ambiguity of this cluster, we considered these three isolates as *Dothiorella* sp. A similar situation was observed for the sequence of the isolate ColPat-449, belonging to the *Diaporthe* genus. The sequence of the ColPat-449 isolate did not match with any specific reference sequences of *Diaporthe* spp. with a high percentage of similarity ($\leq 96\%$) when BLAST searched in GenBank. Consequently, it clustered together with reference sequences of *Dia. chamaeropsis* CBS 753.60 and CBS 454.81 (BS/PP: 88/0.99) in a subclade inside the main clade formed with reference sequences of *Dia. rhusicola*. These isolates did not match with any currently described species belonging to Botryosphaeriaceae or *Diaporthe* spp.; thus, owing to the great advances in fungal taxonomy that have occurred during the last few years, studies to determine their identity to describe them as potential

Table 6. Frequency of reisolation (%) of fungal isolates used in the pathogenicity tests from the inoculated detached and attached shoots, and green fruits of English walnut

Family/fungal species	Isolate	Frequency of reisolation (%) ^z					
		Detached shoots		Attached shoots		Fruit	
		Margin	Margin	2 cm beyond the margin	Margin	Peduncle	
Botryosphaeriaceae							
<i>Botryosphaeria dothidea</i>	ColPat-443	100	66.6	0.0	100	86.0	
<i>Diplodia seriata</i>	ColPat-557	88.0	90.5	0.0	100	71.5	
<i>Dothiorella sarmentorum</i>	ColPat-471	100	59.5	0.0	50.0	0.0	
<i>Dothiorella</i> sp.	ColPat-470	93.0	54.8	0.0	93.0	0.0	
<i>Neofusicoccum mediterraneum</i>	ColPat-556	86.0	54.8	0.0	100	50.0	
<i>Neofusicoccum parvum</i>	ColPat-554	100	95.2	2.4	100	93.0	
Diaporthaceae							
<i>Diaporthe neotheicola</i>	ColPat-448	100	42.9	0.0	100	0.0	
<i>Diaporthe rhusicola</i>	ColPat-444	100	23.8	0.0	100	0.0	
<i>Phomopsis amygdali</i>	ColPat-533	100	42.9	0.0	100	0.0	
Valsaceae							
<i>Cytospora</i> sp.	ColPat-465	81.0	9.5	0.0	50.0	0.0	
Control	PDA	0.0	0.0	0.0	0.0	0.0	

^a Reisolation were done from three shoots or two fruits per experiment (detached shoots, attached shoots or fruits) and fungal isolate combination. For each isolate, two Petri dishes per shoot or fruit and per isolation point were plated with seven wood or fruit pieces per plate. Reisolation percentage was calculated as [(number of positive inoculated points/number of total inoculated points) × 100]. Margin = margin of necrotic lesion.

new species within the *Dothiorella* or *Diaporthe* genera should be conducted in the future.

With respect to our *Cytospora* isolates, their sequences matched many reference sequences of species belonging to the *Cytospora* genus with high similarity; consequently, we were not able to identify them at the species level. Owing to the low importance of this fungi in our study with respect to their frequency in field infections as well as

in aggressiveness (see below), further analyses to infer their identity were not conducted, and they were identified as *Cytospora* sp. As we indicated above, *Cytospora* species have also been associated with branch dieback and shoot blight in other walnut growing areas of the world, such as China, but with low importance (Fan et al. 2015; Zhao et al. 2018).

In general, a pathogenicity test revealed that Botryosphaeriaceae were the most aggressive fungi to walnut in all tissues evaluated (detached and attached shoots and fruit), followed by *Diaporthe* spp. and *Cytospora* sp.; *N. parvum* was the most aggressive among the remaining species tested in any tissues evaluated, followed by *B. dothidea* or *N. mediterraneum*; and *D. seriata*, *Dot. sarmentorum*, and *Dothiorella* sp. were the least aggressive species within the Botryosphaeriaceae group. *Diaporthe* were less aggressive than Botryosphaeriaceae species, and significant differences in aggressive between the *Diaporthe* isolates were observed depending on the infected tissue. All of our results are in accordance with those obtained by Chen et al. (2014) in California, where *N. parvum* was the most aggressive species to walnut after *L. citricola*. These same authors also described *N. mediterraneum* as highly aggressive and *D. seriata*, *Dot. iberica*, *Dia. neotheicola*, and *Dia. rhusicola* as slightly aggressive to walnut. Finally, *Cytospora* sp. was the least aggressive species, causing minimum cankers and internal discoloration in this study. Similar results were obtained by Moral et al. (2017), who tested *C. pruinosa* in olive by inoculating detached shoots and potted plants of olive. In this study, *C. pruinosa* was the least aggressive species in the detached shoots with respect to *N. mediterraneum*, *Nothophoma quercina*, *Comoclathris incompta*, and *Diaporthe* sp., and it was not pathogenic to potted olive plants. Finally, the

Fig. 8. Relative area under the disease progress curve (RAUDPC; %) on green fruits (epicarp) of English walnut cv. Chandler obtained at 21 days after inoculation with mycelial plugs of representative isolates belonging to the Botryosphaeriaceae (dark gray columns) and the *Diaporthe* (light gray columns) and *Cytospora* (white column) genera. Columns represent the mean values of 30 replicate fruits. Columns with common letters do not differ significantly according to Tukey's HSD test ($\alpha = 0.05$). Vertical lines on the columns are the standard error of the mean.

Fig. 9. The biological cycle of branch dieback and shoot blight of the English walnut adapted to the environmental conditions in southern Spain.

Botryosphaeriaceae isolates were the most consistently reisolated fungi from all inoculated tissues in our study, with *N. parvum* showing the highest percentage of reisolation in any case. Note that the consistency of reisolation of the three groups of fungi was in accordance with respect to the aggressiveness of each isolate observed in the pathogenicity tests. This suggests that the most aggressive species (Botryosphaeriaceae) can survive after infection longer in infected tissues than less aggressive species, such as *Diaporthe* or *Cytospora*.

The results obtained in this study are relevant because we determined the main causal agents of branch dieback and shoot blight of English walnut in Spain. This study represents the first report of *B. dothidea*, *Cytospora* sp., *D. seriata*, *Dia. neotheicola*, *Dia. rhusicola*, *Dot. sarmentorum*, *Dothiorella* sp., *N. mediterraneum*, *N. parvum*, and *Ph. amygdali* causing branch dieback and shoot blight of English walnut in Spain and Europe. Altogether, these results are the first approach to describe the etiology of this complex disease, which is causing a growing concern in our walnut plantations. By understanding the diversity of the fungal species associated with the disease, we will be able to pursue studies on biology, epidemiology, and control to establish optimum management strategies to prevent infections. Special attention must be given to the infection process with respect to pruning wounds as well as the potential interaction between pathogens. Recently, studies conducted in California by Agustí-Brisach et al. (2019b) showed that both Botryosphaeriaceae and *Diaporthe* fungi infect walnut shoots through pruning wounds with higher severity when infections occur in the pith tissue instead of in the bark. Additionally, these same authors suggest that both groups of fungi could establish an antagonistic or synergistic interaction during the wood infection process when coinfections occur in the same tissue (Agustí-Brisach et al. 2019b). These later aspects should be considered for studies of the biology and epidemiology of the disease in Spanish plantations. The information generated in the present study has been helpful for obtaining the first description of the biological cycle of the disease according to the environmental conditions in southern Spain (Fig. 9). This region is characterized by a Mediterranean climate with a rainy spring with moderate temperatures followed by a long dry and hot period during the late spring and summer, which favors the dispersal and infection of trunk pathogens. This life cycle should be used to determine the critical moments with respect to the infection of the pathogens to carry out effective management strategies against the disease. Finally, our results are not enough to determine the distribution of Botryosphaeriaceae and *Diaporthe* populations across Spain. A broad survey extended across the Iberian Peninsula should be conducted in the coming years to determine the fungal diversity of canker pathogens in walnut in Spain. It would be helpful to better understand this complex disease and to establish a possible correlation between fungal populations and the agronomical and environmental conditions of the different walnut growing areas of this country.

Acknowledgments

The authors thank Crisolar and Mañán OPFHs and the private companies Almendras Francisco Morales, Bain (Borges Group), and Todolivo for their collaboration.

Literature Cited

- Agustí-Brisach, C., López-Moral, A., Raya-Ortega, M. C., Franco, R., Roca-Castillo, L. F., and Trapero, A. 2019a. Occurrence of grapevine trunk diseases affecting the native cultivar Pedro Ximénez in southern Spain. *Eur. J. Plant Pathol.* 153:599-625.
- Agustí-Brisach, C., Moral, J., Felts, D., Trapero, A., and Michailides, T. J. 2019b. Interaction between *Diaporthe rhusicola* and *Neofusicoccum mediterraneum* causing branch dieback and fruit blight of English walnut in California, and effect of pruning wounds to the infection. *Plant Dis.* 103:1196-1205.
- Agustí-Brisach, C., Moral, J., Pérez-Rodríguez, M., Roca, L. F., Raya-Ortega, M. C., Romero, J., and Trapero, A. 2017. Etiología y perspectivas de control de los chancros y desecaciones de ramas de olivo causados por hongos. *Phytoma Esp.* 292:54-60.
- Analytical Software. 2013. Statistix10 User's Manual. Analytical Software, Tallahassee, FL.
- Armengol, J., Vicent, A., Torné, L., García-Figueres, F., and García-Jiménez, J. 2001. Fungi associated with esca and grapevine declines in Spain: A three-year survey. *Phytopathol. Mediterr.* 40:325-329.
- Aroca, A., García-Figueres, F., Bracamonte, L., Luque, J., and Raposo, R. 2006. A survey of trunk pathogens within rootstocks of grapevines in Spain. *Eur. J. Plant Pathol.* 115:195-202.
- Aroca, A., Raposo, R., Gramaje, D., Armengol, J., Martos, S., and Luque, J. 2008. First report of *Lasiodiplodia theobromae* associated with decline of grapevine rootstock mother plants in Spain. *Plant Dis.* 92:832.
- Barnett, H. L., and Hunter, B. B. 1998. *Illustrated Genera of Imperfect Fungi*, 4th Ed. APS Press, St. Paul, MN.
- Bienapfl, J. C., and Balci, Y. 2013. Phomopsis blight: A new disease of *Pieris japonica* caused by *Phomopsis amygdali* in the United States. *Plant Dis.* 97:1403-1407.
- Campbell, C. L., and Madden, L. V. 1990. *Introduction to Plant Disease Epidemiology*. Wiley, New York, NY.
- Carbone, I., and Kohn, L. M. 1999. A method for designing primer sets for speciation studies in filamentous ascomycetes. *Mycologia* 91:553-556.
- Chen, S. F., Morgan, D. P., Hasey, J. K., Anderson, K., and Michailides, T. J. 2014. Phylogeny, morphology, distribution, and pathogenicity of Botryosphaeriaceae and Diaporthaceae from English walnut in California. *Plant Dis.* 98:636-652.
- Daniels, D. A., Nix, K. A., Wadl, P. A., Vito, L. M., Wiggins, G. J., Windham, M. T., Ownley, B. H., Lambdin, P. L., Grant, J. F., Merten, P., Klingeman, W. E., and Hadziabdic, D. 2016. Thousand cankers disease complex: A forest health issue that threatens *Juglans* species across the U.S. *Forests* 7:260.
- Dhingra, O. D., and Sinclair, J. B. 1995. *Basic Plant Pathology Methods*, 2nd Ed. CRC Press, Boca Raton, FL.
- Díaz, G. A., Latorre, B. A., Ferrada, E., Gutiérrez, M., Bravo, F., and Lolas, M. 2018. First report of *Diplodia mutila* causing branch dieback of English walnut cv. Chandler in the Maule Region, Chile. *Plant Dis.* 102:1451.
- Diogo, E. L. F., Santos, J. M., and Phillips, A. J. L. 2010. Phylogeny, morphology and pathogenicity of *Diaporthe* and *Phomopsis* species on almond in Portugal. *Fungal Divers.* 44:107-115.
- Dissanayake, A. J., Phillips, A. J. L., Li, X. H., and Hyde, K. D. 2016. *Botryosphaeriaceae*: Current status of genera and species. *Mycosphere* 7:1001-1073.
- Fan, X. L., Hyde, K. D., Liu, M., Liang, Y. M., and Tian, C. M. 2015. *Cytospora* species associated with walnut canker disease in China, with description of a new species *C. gicalocus*. *Fungal Biol.* 119:310-319.
- Felsenstein, J. 1985. Confidence limits on phylogenies: An approach using the bootstrap. *Evolution* 39:783-791.
- Glass, N. L., and Donaldson, G. 1995. Development of primer sets designed for use with PCR to amplify conserved genes from filamentous ascomycetes. *Appl. Environ. Microbiol.* 61:1323-1330.
- Gomes, R. R., Glienke, C., Videira, S. I. R., Lombard, L., Groenewald, J. Z., and Crous, P. W. 2013. *Diaporthe*: A genus of endophytic, saprobic and plant pathogenic fungi. *Persoonia* 31:1-41.
- Gramaje, D., Agustí-Brisach, C., Pérez-Sierra, A., Moralejo, E., Olmo, D., Moster, L., Damm, U., and Armengol, J. 2012. Fungal trunk pathogens associated with wood decay of almond trees on Mallorca (Spain). *Persoonia* 28:1-13.
- Guarnaccia, V., Groenewald, J. Z., Woodhall, J., Armengol, J., Cinelli, T., Eichmeier, A., Ezra, D., Fontaine, F., Gramaje, D., Gutierrez-Aguirregabiria, A., Kaliterna, J., Kiss, L., Lignon, P., Luque, J., Mugnai, L., Naor, V., Raposo, R., Sándor, E., Váczy, K. Z., and Crous, P. W. 2018. *Diaporthe* diversity and pathogenicity revealed from a broad survey of grapevine diseases in Europe. *Persoonia* 40:135-153.
- Hau, B., and Kranz, J. 1990. Mathematics and statistics for analyses in epidemiology. Pages 15-21 in: *Epidemics of Plant Diseases*. J. Kranz, ed. Springer-Verlag, Berlin, Germany.
- International Nut and Dried Fruit Council. 2017. <https://www.nutfruit.org/industry/statistics>
- Kornerup, A., and Wanscher, J. H. 1963. *Methuen Handbook of Colour*. Methuen and Co., London, U.K.
- Kumar, S., Stecher, G., and Tamura, K. 2016. MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. *Mol. Biol. Evol.* 33:1870-1874.
- Lawrence, D. P., Travadon, R., Pouzoulet, J., Rolshausen, P. E., Wilcox, W. F., and Baumgartner, K. 2017. Characterization of *Cytospora* isolates from wood cankers of declining grapevine in North America, with the descriptions of two new *Cytospora* species. *Plant Pathol.* 66:713-725.
- Li, G. Q., Liu, F. F., Li, J. Q., Liu, Q. L., and Chen, S. F. 2015. Characterization of *Botryosphaeria dothidea* and *Lasiodiplodia pseudotheobromae* from English walnut in China. *J. Phytopathol.* 164:348-353.
- López-Larrinaga, F., Mellado-Bermejo, E., Valadares de Queirós, R., Figueras-Lorenzo, M., and Gomes-Pires, J. 2017. Las nuevas plantaciones de nogal en España. *Fruticultura* 64:45-59.
- López-Moral, A., Raya-Ortega, M. C., Agustí-Brisach, C., Roca, L. F., Lovera, M., Luque, F., Arquero, O., and Trapero, A. 2017. Morphological, pathogenic, and molecular characterization of *Colletotrichum acutatum* isolates causing almond anthracnose in Spain. *Plant Dis.* 101:2034-2045.
- Lovera, M., Rodríguez, R. A., Arquero, O., and Trapero, A. 2017a. Lesiones necróticas en parte aérea de nogal I. Caracterización y etiología. *Fruticultura* 64:70-89.
- Lovera, M., Rodríguez, R. A., Arquero, O., and Trapero, A. 2017b. Lesiones necróticas en parte aérea de nogal II. Factores que influyen en su incidencia y severidad. *Fruticultura* 64:90-99.

- Martin, M. T., and Cobos, R. 2007. Identification of fungi associated with grapevine decline in Castilla y León (Spain). *Phytopathol. Mediterr.* 46:18-25.
- Melgarejo, P., García-Jiménez, J., Jordá, M. C., López, M. M., Andrés, M. F., and Duran-Vila, N. 2010. *Patógenos de Plantas Descritos en España*, 2nd Ed. Ministerio de Medio Ambiente y Medio Rural y Marino (MARM), Madrid, Spain.
- Michailides, T. J., and Hasey, J. 2010. *Botryosphaeria* and *Phomopsis* cankers of walnuts in California. Walnut Husk Fly Field Meeting. University of California Cooperative Extension, Half Moon Bay, CA.
- Ministerio de Agricultura, Pesca y Alimentación. 2017. <https://www.mapa.gob.es/es/estadistica/temas/estadisticas-agrarias/agricultura/superficies-producciones-anuales-cultivos/>
- Moral, J., Agustí-Brisach, C., Pérez Rodríguez, M., Xavier, C., Raya, M. C., Rhouma, A., and Trapero, A. 2017. Identification of fungal species associated with branch dieback of olive and resistance of table cultivars to *Neofusicoccum mediterraneum* and *Botryosphaeria dothidea*. *Plant Dis.* 101: 306-316.
- Moral, J., Morgan, D., Trapero, A., and Michailides, T. J. 2019. Ecology and epidemiology of diseases of nut crops and olives caused by Botryosphaeriaceae fungi in California and Spain. *Plant Dis.* 103:1809-1827.
- Moral, J., Muñoz-Díez, C., González, N., Trapero, A., and Michailides, T. J. 2010. Characterization and pathogenicity of Botryosphaeriaceae species collected from olive and other hosts in Spain and California. *Phytopathology* 100: 1340-1351.
- Mostert, L., Crous, P. W., Kang, J. C., and Phillips, A. J. L. 2001. Species of *Phomopsis* and a *Libertella* sp. occurring on grapevines with specific reference to South Africa: Morphological, cultural, molecular and pathological characterization. *Mycologia* 93:146-167.
- Norphanphoun, C., Doilom, M., Daranagama, D. A., Phookamsak, R., Wen, T. C., Bulgakov, T. S., and Hyde, K. D. 2017. Revisiting the genus *Cytospora* and allied species. *Mycosphere* 8:51-97.
- O'Donnell, K., and Cigelnik, E. 1997. Two divergent intragenomic rDNA ITS2 types within a monophyletic lineage of the fungus *Fusarium* are nonorthologous. *Mol. Phylogenet. Evol.* 7:103-116.
- Olmo, D., Gramaje, D., and Armengol, J. 2017. Hongos asociados a las enfermedades de la madera del almendro en la Isla de Mallorca. *Fruticultura* 54:18-29.
- Phillips, A. J. L., Alves, A., Abdollahzadeh, J., Slippers, B., Wingfield, M. J., Groenewald, J. Z., and Crous, P. W. 2013. The Botryosphaeriaceae: Genera and species known from culture. *Stud. Mycol.* 76:51-167.
- Phillips, A. J. L., Alves, A., Pennycook, S. R., Johnston, P. R., Ramaley, A., Akulov, A., and Crous, P. W. 2008. Resolving the phylogenetic and taxonomic status of dark-spored teleomorph genera in the Botryosphaeriaceae. *Persoonia* 21:29-55.
- Phillips, A. J. L., Hyde, K. D., Alves, A., and Liu, J. K. 2018. Families in Botryosphaeriales: A phylogenetic, morphological and evolutionary perspective. *Fungal Divers.* 94:1-22.
- Rhouma, A., Triki, M. A., Ouerteni, K., and Mezghanni, M. 2008. Chemical and biological control of *Phomopsis amygdali* the causal agent of constriction canker of almond in Tunisia. *TJPP* 3:69-77.
- Rolshausen, P. E., Úrbez-Torres, J. R., Rooney-Latham, S., Eskalen, A., Smith, R. J., and Gubler, W. D. 2010. Evaluation of pruning wound susceptibility and protection against fungi associated with grapevine trunk diseases. *Am. J. Enol. Vitic.* 61:113-119.
- Ronquist, F., Teslenko, M., Van Der Mark, P., Ayres, D. L., Darling, A., Höhna, S., Larget, B., Liu, L., Suchard, M. A., and Huelsenbeck, J. P. 2012. MrBayes 3.2: Efficient Bayesian phylogenetic inference and model choice across a large model space. *Syst. Biol.* 61:539-542.
- Sánchez, M. E., Venegas, J., and Romero, M. A. 2003. *Botryosphaeria* and related taxa causing oak canker in Southwestern Spain. *Plant Dis.* 87: 1515-1521.
- Sánchez-Solana, J., Sánchez, M. E., Roca, L. F., Raya, M. C., Agustí-Brisach, C., Arquer, O., and Trapero, A. 2018. Enfermedades del castaño causadas por hongos en Andalucía. *Fruticultura* 64:22-43.
- Steel, R. G. D., and Torrie, J. H. 1985. *Bioestadística*, 2nd Ed. McGraw-Hill, Bogotá, Colombia.
- Swofford, D. L. 2003. PAUP* 4.0b10: Phylogenetic Analysis Using Parsimony (*and other methods). Sinauer Associates, Sunderland, Massachusetts.
- Trouillas, F. P., Úrbez-Torres, J. R., Peduto, F., and Gubler, W. D. 2010. First report of twig and branch dieback of English walnut (*Juglans regia*) caused by *Neofusicoccum mediterraneum* in California. *Plant Dis.* 94:1267.
- Udayanga, D., Castlebury, L. A., Rossman, A. Y., Chukeatirote, E., and Hyde, K. D. 2014. Insights into the genus *Diaporthe*: Phylogenetic species delimitation in the *D. eres* species complex. *Fungal Divers.* 67:203-229.
- Úrbez-Torres, J. R. 2011. The status of Botryosphaeriaceae species infecting grapevines. *Phytopathol. Mediterr.* 50:S5-S45.
- Úrbez-Torres, J. R., and Gubler, W. D. 2009. Pathogenicity of Botryosphaeriaceae species isolated from grapevine cankers in California. *Plant Dis.* 93:584-592.
- Úrbez-Torres, J. R., Leavitt, G. M., Voegel, T. M., and Gubler, W. D. 2006. Identification and distribution of *Botryosphaeria* spp. associated with grapevine cankers in California. *Plant Dis.* 90:1490-1503.
- Úrbez-Torres, J. R., Peduto, F., Vossen, P. M., Krueger, W. H., and Gubler, W. D. 2013. Olive twig and branch dieback: Etiology, incidence, and distribution in California. *Plant Dis.* 97:231-244.
- Voglmayr, H., Castlebury, L. A., and Jaklitsch, W. M. 2017. *Juglanconis* gen. nov. on *Juglandaceae*, and the new family *Juglanconidaceae* (*Diaporthales*). *Persoonia* 38:136-155.
- White, T. J., Bruns, T., Lee, S., and Taylor, J. 1990. Amplification and direct sequencing of fungi ribosomal RNA genes for phylogenetics. Pages 315-322 in: *PCR Protocols: A Guide to Methods and Applications*. M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White, eds. Academic Press, San Diego, CA.
- Zhao, S. Z., Guo, K. F., He, L., and Yiming, A. 2018. First report of *Cytospora nivea* causing Cytospora canker on walnut (*Juglans regia* L.) in the Tianshan Mountains Region of Xinjiang, China. *Plant Dis.* 102:2640.