Identification of Fungal Species Associated with Branch Dieback of Olive and Resistance of Table Cultivars to *Neofusicoccum mediterraneum* and *Botryosphaeria dothidea*

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Abstract

Over two consecutive seasons, 16 olive orchards with trees exhibiting dieback symptoms on branches were surveyed in southern Spain. The six dominant fungal species recovered were characterized by means of phenotypic observations, DNA analysis (by sequencing of the internal transcribed spacer, β -tubulin, and large subunit nuclear ribosomal DNA regions), and pathogenicity tests. Additionally, three isolates collected from Tunisian olive trees showing similar dieback symptoms, one isolate of *Colletotrichum godesiae*, and a reference isolates of *Neofusicoccum mediterraneum* were included. The resistance of the 11 most important table cultivars to *N. mediterraneum* and *Botryosphaeria dothidea*, the causal agent of “escudete” (small shield) of fruit, was studied by the inoculation of branches and immature fruit, respectively. The species *Cytospora pruinos*,

N. mediterraneum, *Nothophoma quercina*, *Comoclathris incompta*, and *Diaporthe* sp. were identified. Only *N. mediterraneum* and *C. incompta* were able to induce the typical dieback symptoms and cankers that affected the development of the plants. The species *N. mediterraneum* was the most virulent among the evaluated species, although differences in virulence among its isolates were observed. The remaining fungal species were weakly pathogenic to nonpathogenic on plants. According to resistance tests, ‘Gordal Sevillana’ and ‘Manzanilla Cacereña’ were the most susceptible to branch dieback caused by *N. mediterraneum*. Furthermore, the fruit of ‘Aloreña de Atarfe’ and ‘Manzanilla de Sevilla’ were the most susceptible to *B. dothidea*. Knowledge of the etiology and cultivar resistance of these diseases will help to establish better control measures.

Cultivated olive (*Olea europaea* subsp. *europaea* L.) is the most important perennial crop in Spain. Spain leads the world in olive production, generating about 43% of the world’s olive fruit. The Spanish olive industry accounts for 25% of the global acreage designated for olive production, occupying 2.5×10^6 ha (for both table fruit and oil). Approximately 65% of this land lies in the Andalusia region of the southern Iberian Peninsula, in itself producing 85% of the total production for Spain (Barranco et al. 2008). Global table fruit production is around 2.3×10^6 tons per year, 23% of which come from Spain (MAGRAMA 2016). The most important table cultivars are ‘Gordal Sevillana’ and ‘Manzanilla de Sevilla’, both comprising approximately 85% of table fruit production. In addition, other table cultivars such as ‘Aloreña’ and ‘Morona’ have a secondary importance but their fruit are appreciated for their organoleptic properties. Finally, some cultivars such as ‘Hojiblanca’, ‘Manzanilla Cacereña’, and ‘Verdial de Huévar’ have a twofold purpose as both table fruit and as oil, while some other cultivars such as ‘Picual’ are used exclusively for oil production (Rallo et al. 2005).

At the beginning of the 2000s, a serious disease showing typical dieback symptoms was observed in olive orchards in the Andalusia region, where ‘Gordal Sevillana’ was the most affected cultivar (Romero et al. 2005). Similar symptoms were also observed affecting other cultivars such as ‘Arbequina’, ‘Manzanilla de Sevilla’, and ‘Picual’. Differences in susceptibility among cultivars to the disease are evident in the field but knowledge regarding the resistance of cultivars to twig-branch dieback is unknown. Affected trees showed an

abundance of dead twigs and wilted leaves that remained attached to blighted branches, which were generally associated with the decline of entire young stems or older branches (Moral et al. 2010; Romero et al. 2005; Úrbez-Torres et al. 2013). The branches showing cankers are less tolerant to water stress and had an insufficient water and nutrient flow through both xylem and phloem vessels. Consequently, characteristic dieback symptoms such as bud mortality, leaf chlorosis, fruit rot, and twig dieback occur when water and nutrient demand exceeds the conductive capacity of the vascular tissues (Úrbez-Torres et al. 2013).

Olive fruit can be affected by numerous species of the family Botryosphaeriaceae, including species belonging to the genera *Botryosphaeria* and *Neofusicoccum* that are well known to cause cankers, dieback, and rot of mature and immature fruit (Carlucci et al. 2013; Lazzizzera et al. 2008a, 2008b; Moral et al. 2008a, 2010; Úrbez-Torres et al. 2013). However, further studies may elucidate the etiology of these diseases in Spain, and the relationship between affected host tissue and pathogen species.

The species *N. luteum* (Pennycook & Samuels) Crous, Slippers & A. J. L. Phillips was described causing stem cankers and tip dieback in olive branches in New Zealand (Taylor et al. 2001), and causing leaf necrosis in Australia (Sergeeva et al. 2009). In Spain, Moral et al. (2010) observed that *N. mediterraneum* Crous, M. J. Wingf. & A. J. L. Phillips was the prevalent species associated with branch dieback, mainly on ‘Gordal Sevillana’. The Botryosphaeriaceae species *Diplodia mutila* (Fr.) Mont.; *D. seriata* De Not.; *Dothiorella iberica* A. J. L. Phillips, J. Luque & A. Alves; *Lasiodiplodia theobromae* (Pat.) Griffon & Maubl.; *N. luteum*; *N. parvum*; and *N. vitifusiforme* (Van Niekerc & Crous) Crous, Slippers & A. J. L. Phillips were also reported to cause branch dieback and blight, and eventual death of shoots in different olive-growing areas (Carlucci et al. 2013; Moral et al. 2010; Úrbez-Torres et al. 2013).

On olive drupes, *Diplodia olivorum* A. J. L. Phillips, Frisullo & Lazzizzera and *D. seriata* were reported causing rot on mature fruit in Italy (Lazzizzera et al. 2008a) and Spain (Moral et al. 2008b), respectively. In Australia, *N. luteum* was also observed causing rot of mature fruit (Sergeeva et al. 2009). Conversely, because immature

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olive fruit are highly resistant to fungi, no significant pathogens have been observed causing rot on them, with the exception of *Botryosphaeria dothidea* (Moug.) Ces. & De Not. (Moral et al. 2008b, 2010). This pathogen is the causal agent of Dalmatian disease of olive, which can be found in most of the Mediterranean basin (Iannotta et al. 2007; Latinović et al. 2013; Moral et al. 2010; Zachos and Tzavella-Klonari 1983). *B. dothidea* produces a sunken, necrotic, and circular lesion (approximately 8 mm in diameter, never larger than 1 cm) with a sharp edge delineating infected and healthy fruit tissues; this appearance is called “escudete” (or small shield) in Portuguese and Spanish. As the disease progresses, the necrotic spots expand and eventually encompass the entire fruit. Then, when the fruit matures, it falls to the ground and is mummified (Moral et al. 2010). Overall, the incidence of olive fruit showing the Dalmatian symptoms is relatively low but, frequently, it exceeds the tolerance level for the “Extra” Class standard for the olive fruit, which is usually at a 2 or 4% level, according to the Spanish Royal Decree 1230/2001 or the “Codex Alimentarius” of the FAO, respectively.

Botryosphaeriaceae asexual stage is predominant in the majority of its hosts. In olive, the sexual stage has been reported in only a few cases for *B. dothidea* and *N. mediterraneum* in California and Spain, respectively (Moral et al. 2010, 2015). Recently, Eldesouki (2013) elucidated the Dalmatian disease cycle, in which the mosquito *Prolasioptera berlasiana*, a predator of olive fly eggs (*Bactrocera oleae*), carries the *B. dothidea* spores in a mycangium (a specialized structure, like a pocket). The mosquito is attracted to ovipositor punctures on the olive surface made by the olive fly. When the mosquito deposits its own egg adjacent to the fly egg, it also inoculates the puncture with the fungus. Because the mosquito cannot penetrate intact fruit, the control of olive flies incidentally involves the control of the Dalmatian disease (Moral et al. in press). However, there is little information on the control of Dalmatian disease by the use of resistant olive cultivars. In Montenegro, Latinović et al. (2013) studied the resistance of olive cultivars infected with *B. dothidea*, demonstrating that ‘Crnjaka’ and ‘Gloginja’ (native cultivars) and ‘Pendolino’ and ‘Cassanese’ (Italian cultivars) were highly resistant to the disease. These authors evaluated the resistance of only one Spanish cultivar, ‘Manzanilla’; however, the identity of this cultivar is unclear because the name “Manzanilla” includes more than 20 cultivars, such as ‘Manzanilla de Sevilla,’ ‘Manzanilla Cacerña,’ and so on (Barranco and Rallo 2000). Even so, the resistance of a broad range of Spanish olive cultivars to *B. dothidea* and *N. mediterraneum* has not been evaluated yet in Spain.

During the last few decades, other pathogens have also been associated with olive twig-branch dieback. For example, the species *Cytospora oleina* Berl. and *Eutypa lata* (Pers.) Tul. & C. Tul. have been associated with olive cankers and consequent branch dieback in Greece (Rumbos 1988, 1993). The latter pathogen was recently reported in California and Italy causing dieback of olive trees (Tosi and Natalini 2009; Úrbez-Torres et al. 2013). The species *Comoclathris incompta* (Sacc. & Martelli) Ariyaw. & K. D. Hyde has also been described causing branch dieback in olive in Croatia, Greece, and Italy (Ivic et al. 2010; Malathrakakis 1979; Tosi and Zazzerini 1994). Recently, *Phoma fungicola* has been associated with branch dieback of olive trees in Tunisia (Rhouma et al. 2010; Taieb et al. 2014). In addition, several fungi, including *Diaporthe viticola* Nitschke; *Diatrype oregonensis* (Wehm.) Rappaz; *D. stigma* (Hoffm.) Fr.; *Phaeoacremonium aleophilum* W. Gams, Crous, M. J. Wingf. & Mugnai; *Phaeomoniella chlamydospora* (W. Gams, Crous, M. J. Wingf. & Mugnai) Crous & W. Gams; *Phoma* sp.; *Phomopsis* (Sacc.) Bubák sp. (synonym *Diaporthe* Nitschke); *Trametes versicolor* (L.) Lloyd; and *Schizophyllum commune* Fr., have also been associated with olive branch dieback, stem canker, and twig necrosis in California (Úrbez-Torres et al. 2013). In southern Italy, *N. parvum*; *Pleurostomophora richardsiae* (Nannf.) L. Mostert, W. Gams & Crous; six species belonging to genus *Phaeoacremonium*; and *Pseudophaeomoniella oleicola* Nigro, Antelmi & Crous were isolated from the wood of olive trees displaying wilting and branch dieback (Carlucci et al. 2013, 2015; Crous et al. 2015). *Colletotrichum* spp., the causal agents of olive anthracnose, have also been implicated as responsible for leaf

wilting and branch dieback symptoms (Moral et al. 2014). Although these fungi affect fruit, preliminary studies demonstrated that the toxic substances produced by *Colletotrichum* spp. on rotten fruit are able to incite leaf wilting and branch dieback (Moral et al. 2009). In the latter study, for example, olive plants without fruit were infected by the pathogen but did not show any disease symptoms. However, the plant inoculations were conducted by using a conidial suspension and not using the conventional mycelium plugs of the pathogen (Moral et al. 2009).

In Spain, little attention has been given to olive branch dieback or the Dalmatian disease. Nevertheless, both diseases have increasingly become concerns among growers in the main table-olive-producing areas of the Andalusia region. Determination of the etiology, epidemiology, and control of these diseases are required; therefore, the objectives of the current study were to (i) identify on the basis of morphology and molecular phylogeny the different fungal species associated with the olive twig-branch syndrome, (ii) evaluate the pathogenicity of the different fungi associated with the olive twig-branch dieback, and (iii) evaluate the resistance of the most common table cultivars to *N. mediterraneum* and *B. dothidea*, the causal agents of twig-branch dieback and Dalmatian disease, respectively.

Material and Methods

Field surveys and fungal isolation. In 2009 and 2010, field surveys were conducted in orchards of ‘Gordal Sevillana’ and ‘Picual’ olive located in the Andalusian provinces of Jaén and Sevilla (Table 1). In total, 163 samples were collected from 16 olive orchards, each being 25 years old or older. Branches and twigs from olive trees showing the typical symptoms of branch dieback such as cankers, internal wood necrosis, black vascular streaking, or discolored tissues were collected. Parafilm (Parafilm, Menasha, WI) was used to seal both ends of all samples; then, samples were placed in black plastic bags and kept at 4°C until isolations were made.

Branches and twigs were surface disinfected with 50% ethanol. The outer bark was removed with a sterile scalpel. Small pieces from the edge between healthy and affected wood tissue were plated on potato dextrose agar (PDA; Biokar-Diagnostics, Zac de Ther, France) acidified with lactic acid (2.5 ml at 25% [vol/vol] per liter of medium). Petri dishes were incubated at 23 to 27°C under a 12-h photoperiod of cool fluorescent light (350 $\mu\text{mol m}^{-2} \text{s}^{-1}$) until colonies were large enough to be examined. To obtain pure cultures, mycelial fragments from the margin of the fungal colonies were transferred to acidified PDA and incubated as described before. Several isolates collected in affected ‘Meski’ orchards from Tunisia were also included in this study (Table 1). Fungal species were isolated for further study from 10 olive trees from three different orchards. The studied isolates are maintained in the collection of the Departamento de Agronomía, Universidad de Córdoba, Spain.

Morphological characterization. Based on morphological characteristics, fungal species were first identified to genus. Pycnidial and conidial characteristics such as shape, size, color, and presence or absence of septa were recorded from the colonies developed in vitro. For morphological identification, single conidial cultures were derived using the serial dilution method (Dhingra and Sinclair 1995) and grown on PDA for up to 2 weeks at $25 \pm 2^\circ\text{C}$ with a 12-h photoperiod of cool fluorescent light (350 $\mu\text{mol m}^{-2} \text{s}^{-1}$). To induce pycnidia development, the same isolates were cultured on sterile ‘Gordal Sevillana’ leaves, which were previously autoclaved at 120°C for 20 min, and placed into petri dishes containing 5 ml of molten PDA (Moral et al. 2009). Petri dishes were incubated as described above for 10 days. Three petri dishes per isolate were used. The experiment was performed twice.

Fungal structures were measured using a Nikon Eclipse 80i microscope and images were captured using the NIS-Element software (Nikon Corp., Tokyo). Conidial masses were observed from pycnidia mounted in water. Color (using a color scale) (Kornerup and Wanscher 1963), morphology, and diameter of pycnidia from these isolates were recorded. For conidial measurements, mycelial plugs were removed from the petri dishes, placed on slides with a drop of 0.005% acid fuchsin in lactoglycerol (1:1:1 lactic acid, glycerol, and water),

and covered. For each isolate, 30 measurements were obtained for each structure. The averages of length and width and the length/width relation were calculated. Characteristics of conidia (color, shape, and septation) and mycelia (texture, density, color, and zonation) were also recorded after 14 days of incubation at 23 to 27°C in the dark (Barnett and Hunter 1998; Sutton 1980).

DNA extraction, amplification, and phylogenetic analysis. One to three isolates were selected based on their morphology as representative of each of three fungal taxa, and their identification was confirmed by using molecular techniques (Table 1). Fungal isolates were grown on PDA for 14 days. FastDNA Kit (BIO 101, Inc., Vista, CA) was used to extract total genomic DNA. The nuclear ribosomal DNA (nrDNA) repeats, including the internal transcribed spacer (ITS) 1, 5.8S ribosomal RNA (rRNA), ITS5, and portions of the genes encoding both small and large subunit rRNA, were amplified using primers ITS1 and ITS4 (White et al. 1990). The partial large subunit nrDNA (LSU) was amplified with the primer pair LR0R and LR7 (Rehner and Samuels 1994; Vilgalys and Hester 1990). Oligonucleotide primers Bt2a and Bt2b were used to amplify a portion of the β -tubulin (BT) gene (Glass and Donaldson 1995). Finally, amplification of part of the translation elongation factor 1- α (EF) gene was done with the primers EF1-728F and EF1-986R (Carbone et al. 1999). The polymerase chain reaction (PCR) of BT, EF, ITS, and LSU was conducted according to previous studies (Chen et al. 2015; Lazzizzera et al. 2008b; Moral et al. 2010). The PCR products were purified with an Ultra Clean PCR Clean-Up Kit (MO BIO Laboratories, Inc., Solana Beach, CA). The resulting amplicons were sequenced in both directions using an automated sequencer by the University of Córdoba sequencing facility (ABI Prism 3130XL Genetic Analyzer; Applied Biosystems, Foster City, CA). The nucleotide sequences were read and edited with FinchTV 1.4.0. Sequences were assembled and edited to resolve ambiguities using the program SeqMan (DNASTAR Lasergene, Madison, WI), and consensus sequences for all isolates were compiled into a single file (Fasta format).

Phylogenetic analysis with all isolates was first conducted with the BT, EF, ITS, and LSU data sets individually, and the four topologies were compared. A partition homogeneity test was performed to determine whether BT, EF, ITS, and LSU data sets could be combined. The combined alignment of the two loci BT and ITS was first analyzed for inferring the phylogeny of the isolates. Phylogenetic analysis on the LSU single-locus alignment was used to elucidate the organismal phylogeny. GenBank sequences (Table 1) from different species of *Neofusicoccum*, *Colletotrichum*, *Cytospora*, *Phoma*-like, or *Diaporthe* were selected based on their high similarity with our query sequences using MegaBLAST. *Ganoderma resinaceum* (GR145) and *Coltricia cinnamomea* (Dai 2464) were used as the outgroups of the combined alignment of the two loci BT and ITS, and the LSU single-locus alignment. These were added to the sequences obtained and aligned using CLUSTALW v. 2.0.11 (Larkin et al. 2007). The genetic distances were calculated using the Kimura two-parameter model. For phylogenetic inference, the neighbor-joining (NJ) method was used (Saitou and Nei 1987). The NJ tree and the statistical confidence of a particular group of sequences in the tree, evaluated by bootstrap (BS) test (2,000 pseudoreplicates), were performed using the computer program MEGA 6 (Tamura et al. 2013). Sequences derived in this study were lodged at GenBank. GenBank accession numbers of the strains collected during this study are listed in Table 1.

Pathogenicity tests. One representative isolate of each identified species was inoculated toward to determine their pathogenicity capacity. In addition, one *Colletotrichum godetiae* isolate (CH-21), which belongs to the *C. acutatum* species complex and is the dominant species in the Andalusian population of the pathogen (Moral et al. 2014), was selected to determine pathogenicity on olive detached branches or potted plants (Table 1).

Pathogenicity on detached branches. In March 2010, branch segments (14 to 18 cm long and 1.0 to 1.5 cm in diameter) were collected from 'Gordal Sevillana' trees from The Olive Cultivars Garden orchard that belongs to the Andalusian Institute for Research and Formation in Agriculture and Fishery (IFAPA in Spanish) and is located in Mengíbar (Jaén province, Andalusia region). The branch segments

were sealed at both ends with Parafilm to reduce desiccation, and bark surfaces were sterilized with 70% ethanol at the point of inoculation, situated in the middle of each branch. In order to remove the outer bark yet leave the inner bark intact, 10-mm-diameter holes were made in the bark using a cork borer. In the cavities, a 10-mm-diameter mycelium plug of each isolate was inserted such that the inoculum was placed in direct contact with vascular tissues, as described by Moral et al. (2010); then, the inoculated area was wrapped with Parafilm. In total, 11 representative isolates belonging to six species were individually tested using five replicate branches per isolate. The experiment was repeated three times and five branches were treated with acidified PDA as control. Inoculated and control branches were then incubated in humid chambers (plastic containers, 30 by 23 by 6 cm, with 100% relative humidity [RH] obtained by adding 300 ml of water) at room temperature (26 to 33°C) under continuous cool fluorescent lights (350 $\mu\text{mol m}^{-2} \text{s}^{-1}$) for 30 days. Humid chambers were placed inside at room temperature in a completely randomized design. For each branch, evaluation of the lesion length and the length of the colonized bark surface bearing pycnidia, and calculation of the relative (percent) affected length of each branch were obtained according to Moral et al. (2010). Small wood fragments that form the margin of the affected area of each inoculated branch were plated on acidified PDA to isolate the pathogens.

Pathogenicity on potted plants. Five-year-old potted 'Gordal Sevillana' plants were obtained from a commercial nursery. Each plant was inoculated with mycelium plugs as described above for detached branches. The same 11 isolates were inoculated using three replicate plants per isolate and four branches per plant. After inoculation, plants were placed in a greenhouse at 25 to 30°C in a completely randomized design and were irrigated as needed. The area around the experiment was abundantly irrigated to increase the RH. Lesion length and percentage of dead branches were assessed 90 days after inoculation. The pathogens were isolated from the olive branches as described above. The experiment was repeated three times and five branches from the same plant were treated with acidified PDA as control in each replicate of the experiment.

Evaluation of cultivar resistance. To evaluate the resistance of different table cultivars to *N. mediterraneum*, which was the most virulent species of the previous experiments, branches and 5-year-old plants of 10 table cultivars were inoculated using the reference isolate *N. mediterraneum* BOO071. In the same way, the resistance of cultivars to *B. dothidea* was evaluated using the reference isolate *B. dothidea* BOO046 (Moral et al. 2010).

Branch inoculation with *N. mediterraneum*. In March 2010, branch segments (14 to 18 cm long and 1.0 to 1.5 cm in diameter) were cut from 10 table olive cultivars growing at The Cultivar Garden orchard (Table 2). Sampling and inoculation were conducted as described above. Ten branches per cultivar were inoculated and the experiment was conducted three times. Likewise, another 10 branches per replicate were treated with acidified PDA as control. In total, 270 branches were inoculated with *N. mediterraneum* and 90 branches were used as control. All of the branches were incubated in humid chambers as described above. Evaluation of lesion length and the length of the colonized bark surface bearing pycnidia, and calculation of the relative (percent) affected length of each branch were obtained according to Moral et al. (2010). The pathogen was reisolated from the branches as described above.

Plant inoculation with *N. mediterraneum*. Five-year-old potted plants of seven table cultivars were obtained from a commercial nursery in Córdoba province from Andalusia (Table 2). Each plant was inoculated with *N. mediterraneum* using mycelial plugs as described above. Three replicate plants and four branches of each one per cultivar were inoculated. Five branches from the same plant were treated with acidified PDA as control in each replicate (plant) of the experiment. Inoculated plants were then incubated in a greenhouse from 25 to 30°C in a completely randomized design, and irrigated abundantly to increase the RH. The percentage of dead branches and the lesion length of inoculated branches were assessed 90 days after inoculation. The experiment was repeated four times. The pathogen was isolated from the branches as described above.

Fruit inoculation with *B. dothidea*. In September 2010, immature fruit of 10 table cultivars were collected from The Cultivar Garden orchard (Table 2). Fruit were washed, disinfested, and inoculated according to Moral et al. (2010). Inoculated and control fruit (PDA plugs without mycelia) were incubated in humid chambers as described above. Disease severity was assessed every 2 weeks for 2 months using a 0-to-5 rating scale previously described by Moral et al. (2008a). Disease severity index (DSI) was calculated according to the formula $DSI = (\sum ni \times i) / N$, where i represents severity (0 to 5), ni is the number of fruit with the severity of i , and N is the total number of fruit (Moral et al. 2008a). The area under the disease progress curve (AUDPC) was calculated by trapezoidal integration of DSI values over time. There were three replicates (humid chambers) per treatment and 30 fruit per replicate. Treatments were arranged in a completely randomized design.

Statistical analysis. Analysis of variance (ANOVA) and means comparison tests were conducted to determine the differences in virulence among isolates and in resistance among cultivars. For the different analyses conducted, dependent variables were lesion length to evaluate differences in virulence among isolates; lesion length and pycnidia colonization or dead branches (percent) to evaluate differences in resistance among cultivars by using detached branches or potted plants, respectively; and AUDPC to evaluate differences in resistance among cultivars by using detached fruit. When ANOVA showed significant differences, the treatment means were compared according to Tukey's honestly significant difference test at $P = 0.05$. The nonpathogenic isolates were excluded from the statistical analysis. The data were logarithmically transformed to meet the homogeneity of the variances or normality. Because the data of cultivar resistance to *N. mediterraneum* did not show homogeneity of variances, even with the logarithmic transformation, they were analyzed using the Kruskal-Wallis nonparametric test and the cultivars were compared according to Dunn's test at $P = 0.05$. Linear regression was used to study the relationship between dependent variables of this study. In addition, we studied the relation between fruit resistance to *B. dothidea* and the susceptibility to fruit rot caused by *Colletotrichum* spp. (Moral et al. 2014). All the data were analyzed by using Statistix 10 (Analytical software 2013).

Results

Collection of fungal isolates. In total, 120 fungal isolates from affected olive trees showing twig-branch dieback were recovered in Spanish olive orchards. Overall, olive twig death was usually associated with cankered stems. Among the collected isolates, five fungal groups were clearly differentiated, the most frequent being Botryosphaeriaceae species (mainly *N. mediterraneum*), followed by *Cytospora* spp., a *Phoma*-like species (two subgroups), and *Diaporthe* spp. Representative isolates from each of the five fungal groups were selected for further studies. In addition, a fungal group of isolates that were frequently isolated from affected orchards in Tunisia was included.

Morphological characterization. All studied isolates showed mycelial growth and sporulation within 14 days of incubation on olive leaves, except one *Phoma*-like isolate, CH-12, which showed very poor mycelial growth but developed some pycnidia around the original mycelium plug. Based on their appearance in culture, the isolates were again assigned to five main fungal groups (Table 3). The first group comprised a Botryosphaeriaceae species with a *Neofusicoccum* anamorph (isolates BOO071 and CH-06); this species was characterized by floccose to felted aerial mycelium showing colors that varied from light to olive green on acidified PDA. Conidia were hyaline, aseptate, and fusiform. Pycnidia were dark, ostiolate, globose, and erumpent. Two groups (group 2 and 3) were clearly differentiated, belonging to *Phoma*-like isolates. Group 2 (isolates CH-04 and CH-16) was characterized by brown to yellowish mycelium with a regular margin. Conidia were dark, ellipsoid or ovoid, slightly flattened, and thin-walled. Pycnidia were irregular, branched, wooded, velvety, and sprawled. Group 3 (isolates CH-14 and CH-15) showed flat, fine, medium density to very dense mycelium; the color varied from white to pink, or green to yellow, with a

regular colony margin. Conidia were hyaline, aseptate, and oblong. Pycnidia were dark, ostiolate, and globose. A fourth group included a *Diaporthe* sp. that was characterized by having white, cottony, slow-growing, raised mycelium with a regular colony margin. In this fourth group, two types of hyaline conidia were observed: oblong, slightly fusiform, and ligulated conidia (α); and filiform and curved conidia (β). Pycnidia were dark, ostiolate, globose, erumpent, and velvety. Finally, *Cytospora* isolates formed a fifth group characterized by white, flat, felty, texture uniform mycelium with an irregular colony margin. Conidia were hyaline, aseptate, and with an alantoid form. Pycnidia were dark, erumpent, ostiolate, tuberculate, globous, and velvet. (Tables 3 and 4; Fig. 1).

Molecular characterization. To confirm the identification based on morphology, BT, EF, ITS, and LSU regions were obtained for all isolates studied. The combined alignment of BT and ITS included 18 taxa (including the outgroup *G. resinaceum*) (Fig. 2). In this phylogenetic tree, combined sequences of the genera *Colletotrichum* (isolate CH-21), *Cytospora* (isolate CH-13), *Diaporthe* (isolates CH-01 and CH-03), *Neofusicoccum* (isolates CH-06 and BOO071), and *Nothophoma* (isolates CH-4, CH-14, and CH-15) formed five well-supported clades, in which BS support ranged from 94 to 100%. Each clade incorporated one representative isolate of *C. godetiae* (CBS 127561), *Cytospora pruinosa* (Fr.) Sacc. (CBS 118555), *Diaporthe* sp. (UCR1395), *N. mediterraneum* (UCD720SJ) and *Nothophoma quercina* (Syd. & P. Syd.) Q. Chen & L. Cai (CBS 633.92) for *Colletotrichum*, *Cytospora*, *Diaporthe*, *Neofusicoccum*, and *Nothophoma* clades, respectively. However, the isolates CH-12 and CH-16 clustered in a subclade close to *Leptosphaeria biglobosa* Shoemaker & H. Brun (CBS 532.66) with a low BS value (BS = 74%) and were hypothesized as a *Phoma*-like species. Subsequently, phylogenetic analysis on the LSU single-locus alignment was analyzed for the elucidation of the organismal phylogeny of the doubtful isolates (Fig. 3). This alignment was successful in identifying all of the isolates. The isolates CH-01 and CH-03 were grouped together in a clade (BS = 99%) with a representative isolate of *Diaporthe* sp. (PHAg); the isolate CH-13 clustered (BS = 100%) with a representative isolate of *C. pruinosa* (CBS119207); and the isolates CH-12 and CH-16 clustered (BS = 93 and 100%, respectively) with a representative isolate of *Comoclathris incompta* (CBS 467.76). The rest of the isolates formed the same clades as those observed in the combined alignment of BT and ITS. Finally, sequences of EF and its single-locus alignment were not helpful in identifying our *Phoma*-like isolates.

Pathogenicity tests. *Pathogenicity on detached branches.* Necrotic lesions on 'Gordal Sevillana' branches were observed 2 weeks after inoculation. In general, vascular tissues showed sunken and sectorial necrotic lesions in the affected wood. Eventually, pathogens belonging to the *Cytospora*, *Phoma*-like, or *Diaporthe* genera produced cankers on the bark of the inoculated branches. Only the two *N. mediterraneum* isolates (BOO071 and CH-06), a *Cytospora* sp. isolate (CH-13), and a *C. incompta* isolate (CH-16) caused visible lesions on detached branches. Both *N. mediterraneum* isolates were the most virulent ($P < 0.05$), causing an average length of necrosis of 15.93 ± 3.04 and 13.51 ± 5.96 cm, respectively (Fig. 4). The remaining fungal species (*Colletotrichum godetiae*, *Phoma*-like sp., and *Diaporthe* sp.) did not cause lesions on inoculated branches. Further, no lesions were observed in control branches. The species *N. mediterraneum*, *Cytospora pruinosa*, and *Comoclathris incompta* were isolated from affected tissues, and *Colletotrichum godetiae*, *Diaporthe* sp., and *Nothophoma quercina* were isolated from asymptomatic branches. A significant and positive linear correlation ($P < 0.001$, $R^2 = 0.724$) was observed between the length of necrosis and pycnidia production, the bark surface inoculated with *N. mediterraneum* isolate BOO071 being that producing the highest number of pycnidia of the pathogen (data not shown).

Pathogenicity on potted plants. The first symptoms were observed 30 days after inoculation with mycelium plugs on the xylem tissues of Gordal Sevillana. Symptoms—mainly vascular necrotic lesions—were similar to those observed in detached branches. Only the *N. mediterraneum* isolates BOO071 and CH-06 and the *C. incompta* isolate CH-06 were able to induce the typical dieback

symptoms and cankers that affected the development of the plants, where the BOO071 isolate was the most virulent (Fig. 4). The remaining fungal species (*C. godetiae*, *C. pruinosa*, *Diaporthe* sp., and *N. quercina*) did not cause any lesions on inoculated plants as with the control branches. The species *N. mediterraneum* and *C. incompta* were consistently isolated from affected tissues while *C. godetiae*, *C. pruinosa*, *Diaporthe* sp., and *N. quercina* were isolated distantly from points away from the inoculation point on asymptomatic branches.

Evaluation of cultivar resistance. *Branch inoculation with N. mediterraneum.* Olive branches of the nine inoculated cultivars showed vascular necrotic tissues after 2 weeks of inoculation. Symptoms observed were the same as those described above. According to the lesion on detached and inoculated branches, ‘Gordal Sevillana’ was significantly ($P < 0.05$) the most susceptible to the pathogen (necrosis length 15.72 ± 4.62 cm), followed by ‘Santa Caterina’ and ‘San Agostino’, which did not show significant differences ($P > 0.05$) among them. Conversely, ‘Manzanilla Cacereña’ was the least susceptible cultivar (necrosis length 6.19 ± 2.11 cm), followed by ‘Verdial de Huévar’ and ‘Morona’, which did not show significant ($P > 0.05$) differences among them (Table 2). For all of the cultivars, a weak but significant negative correlation ($R^2 = 0.190$, $P = 0.0001$) between branch diameter and length of necrosis was observed, showing that branch susceptibility increased when the diameter of branches

decreased. Finally, a significant positive linear correlation ($R^2 = 0.1359$, $P < 0.001$) was observed between the length of necrosis and pycnidia production (data not shown), the bark surface of ‘San Agostino’ being the most occupied by pycnidia.

Plant inoculation with N. mediterraneum. Olive plants showed the first symptoms at 14 days after inoculation, while the first dead branches were observed at 8 weeks. In the potted plant trials, ‘Manzanilla Cacereña’ and ‘Gordal Sevillana’ showed a high percentage of dead branches, 83.33 and 62.50%, respectively, whereas ‘Aloreña de Atarfe’, ‘Hojiblanca’, and ‘Verdial de Huévar’ did not produce any dead branches (Table 2). According to necrosis length, ‘Manzanilla Cacereña’ and ‘Gordal Sevillana’ were also the most susceptible to the pathogen (necrosis lengths of 10.6 ± 3.27 and 10.38 ± 2.52 cm, respectively) without significant differences between them. The remaining cultivars (‘Aloreña de Atarfe’, ‘Hojiblanca’, ‘Manzanilla de Sevilla’, ‘Morona’, and ‘Verdial de Huévar’) were significantly less susceptible (necrotic lesions of 3.06 to 5.25 cm) than the previous two cultivars and formed a homogeneous group ($P > 0.05$). When we studied the relationship between the percentage of dead branches of the different cultivars and their canker lengths (from detached branches and branches in vivo), the percentage of dead branches was observed to be significantly ($R^2 = 0.965$, $P < 0.001$) related to the canker lengths of inoculated potted plants, whereas the relationship was not significant ($P = 0.8740$) when detached branches were used.

Fig. 1. Two-week-old colonies on potato dextrose agar of the different fungal species isolated from olive trees in Spain and Tunisia. **A**, *Colletotrichum godetiae* isolate CH-21; **B** and **C**, *Comoclathris incompta* isolates CH-12 and CH-16, respectively; **D**, *Cytospora pruinosa* isolate CH-13; **E** and **F**, *Diaporthe* sp. isolates CH-01 and CH-03, respectively; **G**, *Neofusicoccum mediterraneum* isolate CH-06; **H** to **J**, *Nothophoma quercina* isolates CH-04, CH-14, and CH-15, respectively; **K**, Pycnidia of *N. mediterraneum* isolate CH-06 embedded in the bark of a young olive stem; **L**, Canker lesion caused by *N. mediterraneum* isolate CH-06 on olive branch from San Agostino olive.

Fruit inoculation with B. dothidea. When immature olive fruit were inoculated with *B. dothidea*, the first symptoms were observed at 21 days after inoculation. These were small, depressed, necrotic lesions surrounding the inoculation point that advanced until they covered the entire surface of the fruit. 'Hojiblanca', 'Manzanilla de Sevilla', and 'Morona' olive fruit were the most resistant to *B. dothidea*, although there was an extensive overlap for the AUDPC showed by the fruit of the different cultivars (Table 2). The resistance to *B. dothidea* was not correlated ($P = 0.6968$) with the resistance to fruit rot caused by *C. godetiae*.

Discussion

Six fungal species belonging to six different genera were isolated from olive plants showing branch dieback symptoms in Spain and Tunisia. Affected trees showed defoliation or leaf wilting in young twigs and darker, sunken areas along affected branches, which revealed perennial cankers under the bark. Cankered zones presented a well-defined dark line of demarcation between infected and healthy tissues. Dieback diseases characterized by perennial cankers affecting branches and trunks have been studied in many different

perennial hosts worldwide, including olive (Moral et al. 2010; Úrbez-Torres et al. 2013). The current work elucidates the etiology of olive branch dieback in Spain, with a special emphasis on the pathogenicity of associated fungi. Moreover, the resistance of the main table cultivars against the main causal agents of the disease has also been evaluated.

Morphological characteristics (conidial and mycelial) were useful to separate the isolates into four different groups (Botryosphaeriaceae, *Cytospora*, *Phoma*-like, and *Phomopsis*), in agreement with those described for each genus (Chen et al. 2015; Liu et al. 2015; Phillips et al. 2013; van Niekerk et al. 2004). Nevertheless, taxa of *Phoma*-like species are morphologically difficult to distinguish. This is also true for *Ascochyta* spp. Both of these genera have, in the past, been linked to *Didymella* sexual morphs. Recently, Chen et al. (2015) clarified the generic delimitation in the family Didymellaceae by combining multilocus phylogenetic analyses using ITS, LSU, the RNA polymerase II second largest subunit (*rpb2*) gene, partial gene regions of BT (*tub2*), and morphological observations. LSU, in particular, has been described as one of the most helpful regions to identify

Table 1. Fungal isolates from olive trees located in Spain and Tunisia, and sequences from GenBank used in this study

Species	Isolate ^z	Host, cultivar	Symptoms	Collector	Origin	GenBank Accession number ^y		
						BT	ITS	LSU
<i>Botryosphaeria dothidea</i>	BOO046	<i>Olea europaea</i> Santa Caterina	Dalmatian disease	J. Moral	Mengibar, Jaen, Andalusia, Spain	GU292738	GU292626	n/a
<i>Colletotrichum godetiae</i>	CH-21	<i>O. europaea</i> Gordal Sevillana	Soapy fruit	A. Trapero	Palomera, Cordoba, Andalusia, Spain	KU973701	KU973719	KU973721
	CBS 127561	<i>Ugni molinae</i>	Twig, tip necrosis	U. Damm	Chile, South America	JQ950093	JQ948442	n/a
<i>Coltricia cinnamomea</i>	Dai 2464	...	...	T. Wagner and M. Fischer	Finland	n/a	-n/a	AF311003
<i>Comoclathris incompta</i>	CH-12	<i>O. europaea</i> Picual	Branch canker	J. Moral	Jaen, Jaen, Andalusia, Spain	KU973707	KU973715	KU973728
	CH-16	<i>O. europaea</i> Meski	Branch canker	A. Rhouma	Tunisia	KU973708	KU973716	KU973729
	CBS 467.76	<i>O. europaea</i>	...	M. M. Averskamp	Greece	n/a	n/a	GU238087
<i>Cytospora pruinosa</i>	CH-13	<i>O. europaea</i> Gordal Sevillana	Branch canker	J. Moral	Mengibar, Jaen, Andalusia, Spain	KU973702	KU973711	KU973722
	CBS 118555	<i>O. europaea</i> africana	Branch canker	Y. Li Wang	South Africa	KM034893	DQ243790	n/a
	CBS 119207	...	...	J. Z. Groenewald	...	n/a	n/a	EU552121
<i>Diaporthe</i> sp.	CH-01	<i>O. europaea</i> Gordal Sevillana	Branch canker	A. Trapero	Sevilla, Sevilla, Andalusia, Spain	KU973709	KU973717	KU973730
	CH-03	<i>O. europaea</i> Gordal Sevillana	Branch canker	M. Pérez-Rodríguez	Sevilla, Sevilla, Andalusia, Spain	KU973710	KU973718	KU973731
	UCR1395	<i>Persea americana</i> Hass	Fruit	M. Twizeyimana	San Diego, CA	JX898989	JX869042	n/a
	PHAg	...	Branch canker	M. Pilloti	Rome	n/a	AY620999	AY621002
<i>Ganoderma resinaceum</i>	GRI45	...	...	C. L. Su	Shanghai, China	DQ288101	KC311374	n/a
<i>Leptosphaeria biglobosa</i>	CBS 532.66	<i>Brassica</i> sp.	...	...	The Netherlands	KT389840	KT389541	KT389759
<i>Neofusicoccum mediterraneum</i>	CH-06	<i>O. europaea</i> Gordal Sevillana	Branch canker	J. Moral	Sevilla, Sevilla, Andalusia, Spain	KU973703	KU973712	KU973723
	BOO071	<i>O. europaea</i> Gordal Sevillana	Branch canker	A. Trapero	Arahal, Sevilla, Andalusia, Spain	GU292757	GU292645	KU973724
	UCD720SJ	<i>Vitis vinifera</i>	Branch canker	J. R. Úrbez-Torres	California	GU799475	GU799452	n/a
	CBS 268.80	...	...	...	...	n/a	n/a	AY004336
<i>Nothophoma quercina</i>	CH-04	<i>O. europaea</i> Gordal Sevillana	Branch canker	M. Pérez-Rodríguez	Sevilla, Sevilla, Andalusia, Spain	KU973704	KU973713	KU973725
	CH-14	<i>O. europaea</i> Meski	Branch canker	A. Rhouma	Tunisia	KU973705	KU973714	KU973726
	CH-15	<i>O. europaea</i> Meski	Branch canker	A. Rhouma	Tunisia	KU973706	KU973720	KU973727
	CBS 633.92	<i>Microsphaera alphitoides</i> , <i>Quercus</i> sp.	...	...	Ukraine	GU237609	GU237900	EU754127

^y BT = β -tubulin, ITS = internal transcribed spacer, LSU = large subunit ribosomal RNA, and n/a = not available at the time of this publication.

^z CBS = Centraalbureau voor Schimmecultures, Utrecht, The Netherlands; UCD = University California-Davis; and UCR = University California-Riverside. Sequences from GenBank used in the phylogenetic analysis are indicated in bold.

species belonging to the Didymellaceae family. Here, the BT, ITS, and LSU regions were needed to identify the species of each genus, LSU being especially helpful to identify *Phoma*-like spp. Conversely, sequences of EF were unhelpful for identification. The ITS and EF combined phylogenetic analysis was useful in describing the diversity of species that affect olive trees in Spain and Tunisia, confirming the five described groups based on morphology.

Concerning the Botryosphaeriaceae group, only one species, *N. mediterraneum*, was recovered, the prevalent pathogen in this work. The current study corroborates the presence of this species in lignified tissues of olive trees in Spain, suggested as the main causal agent of the disease (Moral et al. 2010; Romero et al. 2005). In addition, *N. mediterraneum* was the most virulent species from this study, although differences in virulence between tested isolates were observed. These differences were only observed when pathogenicity was evaluated on potted plants; they were not observed on detached branches. This result suggests that there is a possibility of finding genetic diversity within *N. mediterraneum* populations with geographic and host preferences. In fact, the teleomorph of *N. mediterraneum* was reported for the first time in the world in the Andalusian region (Moral et al. 2015), which invites us to further research its genetic diversity. In addition, studies conducted in Spain by Romero (2012), in which olive plants were inoculated with *B. dothidea*, *Diplodia corticola*, *Dothiorella iberica*, and *N.*

mediterraneum, showed that only *N. mediterraneum* was able to induce cankers on plants with a high level of virulence. In other olive-growing areas, a broad range of species belonging to the Botryosphaeriaceae family (*D. mutila*, *D. seriata*, *D. iberica*, *L. theobromae*, *N. luteum*, and *N. parvum*) has been associated with olive trees showing branch dieback (Sergeeva et al. 2009; Taylor et al. 2001; Úrbez-Torres et al. 2013).

Among all of the fungal taxa isolated from the symptomatic wood of olive in this study, *Phoma*-like species were the most diverse. All of the Tunisian isolates in this study were identified as *Phoma*-like species. In concordance with this result, today, only *Phoma* sp. and *Phoma fungicola* have been reported as causing branch dieback of olive trees in this country (Rhouma et al. 2010; Taieb et al. 2014). Our Tunisian isolates were identified as *N. quercina* and *C. incompta* but the latter was pathogenic to both detached branches and potted plants. In agreement with this result, *C. incompta* has also been described as causing branch dieback on olive in Croatia (Ivic et al. 2010), Greece (Malathrakakis 1979), and Italy (Tosi and Natalini 2009; Tosi and Zazzerini 1994). Concerning negative pathogenicity results obtained for the *N. quercina* isolates (both Spanish and Tunisian), previous surveys on olive orchards in California revealed that the occurrence of *Phoma* spp. in olive is very low (Úrbez-Torres et al. 2013), leading us to hypothesize that it has limited pathogenicity in olive. It is well known that the *Phoma* genus contains plant-

Table 2. Susceptibility of olive cultivar inoculated with *Neofusicoccum mediterraneum* on detached branches and potted plants and *Botryosphaeria dothidea* on detached fruit^w

Cultivar	<i>Neofusicoccum mediterraneum</i>				<i>Botryosphaeria dothidea</i>
	Detached branches		Potted plants		Fruit
	Canker (cm)	Pycnidia (cm) ^x	Canker (cm)	Dead (%) ^y	AUDPC ^z
Aloreña de Atarfe	–	–	4.11 b	0.00	1.70 a
Ascolana Tenera	9.52 cd	3.56 bc	n/e	n/e	n/e
Gordal Sevillana	15.72 a	3.03 bcde	10.38 a	62.50	1.12 c
Hojiblanca	11.22 bc	3.19 bcde	3.43 b	0.00	0.59 de
Manzanilla Cacereña	6.19 e	2.74 cde	10.60 a	83.33	0.82 cd
Manzanilla de Sevilla	12.38 b	3.26 bcd	4.80 b	16.67	1.54 ab
Morona	7.64 de	2.23 e	5.25 b	16.67	0.82 cd
Ocal	n/e	n/e	n/e	n/e	1.19 bc
San Agustino	13.21 ab	5.38 a	n/e	n/e	0.29 e
Santa Caterina	14.03 ab	3.63 b	n/e	n/e	1.01 c
Verdial de Huévar	6.32 e	2.24 de	3.06 b	0.00	1.04 c

^w Mean values with the same letter in a row are not significantly different according to Tukey's honestly significant difference test ($P < 0.05$); – indicates that no symptoms were observed and n/e = cultivars not evaluated in each experiment.

^x Pycnidia colonization.

^y Dead branches.

^z Area under the disease progress curve.

Table 3. Morphological characteristics and mycelial growth of *Colletotrichum*, *Cytospora*, *Diaporthe*, *Neofusicoccum*, and *Phoma*-like isolates obtained from olive

Species	Isolate	Mycelia					
		Obverse			Reverse		Growth ^y
		Color	Zonation	Margin	Color	Zonation	
<i>Colletotrichum godetiae</i>	CH-21	Gray	No	Regular	Dark gray	No	6.20
<i>Cytospora</i> sp.	CH-13	White	No	Irregular	White	No	10.69
<i>Diaporthe</i> sp.	CH-01	White	Yes	Regular	White green	Yes	12.86
	CH-03	White	Yes	Regular	White gray	Yes	14.91
<i>Neofusicoccum mediterraneum</i>	CH-06	Light green	No	Regular	Gray Green	No	17.84
	BOO071	Olive green	No	Irregular	Olive green	No	16.37
Phoma-like	CH-04	Brown yellow	Yes	Regular	Brown red	Yes	3.30
	CH-12 ^z	–	–	–	–	–	–
	CH-14	White pink	No	Regular	Orange salmon	No	7.95
	CH-15	White pink	No	Regular	Orange salmon	No	9.28
	CH-16	Green yellow	No	Regular	Dark brown	No	2.27

^y Growth (mm/day). Single conidial cultures were grown on potato dextrose agar for up to 2 weeks at 25 ± 2°C with a 12-h diurnal photoperiod of cool fluorescent light (350 μmol m⁻² s⁻¹).

^z Mycelia of this isolate was not optimum for development for its characterization.

pathogenic species as well as numerous saprobic and endophytic species associated with a wide range of hosts (Chen et al. 2015).

Species in the genus *Diaporthe* were the third most prevalent fungi isolated in our study, with two isolates identified as *Diaporthe* spp. To date, species of this genus have only been associated with olive branch dieback in California (Úrbez-Torres et al. 2013). In our study, pathogenicity tests revealed that all isolates of *Diaporthe* were not pathogenic on olive branches. However, these results contrast with those obtained by Úrbez-Torres et al. (2013), who demonstrated the pathogenicity of some *Diaporthe* isolates on olive branches. Because species of *Diaporthe* are known to be cosmopolitan and found primarily as endophytes, parasites, and saprotrophs in a wide range of hosts (Udayanga et al. 2011), this difference in pathogenicity in olive between both countries could be due to variability in isolate virulence. Many other possible factors such as the age of the host or differences in cultivar susceptibility could also be explanatory. Thus, a broader pathogenicity study using a higher number of isolates of this genus and different plant cultivars will be required to further clarify the pathogenic role of *Diaporthe* spp. as causal agents of branch dieback on olive.

Cytospora pruinosa was isolated with the least prevalence. In addition, this was the least virulent species to detached branches among all the pathogenic fungi tested in this study, and was found to not be pathogenic to the potted plants. To our knowledge, this is the first report of *C. pruinosa* causing olive branch dieback in Spain. To date, only the species *C. oleina* has been associated with olive branch dieback in Greece (Rumbos 1988). Our results suggest that, although many fungi such as *Diaporthe* are pathogenic to a wide range of crops, they would only be able to induce cankers on hosts such as

weakened or stressed olive trees. Notably, no relationship was found between the dead branches (percentage) of inoculated plants and canker length on detached branches. This reinforces the idea that inoculation in vivo is essential to the characterization of fungal pathogens or even to identify differences in virulence among isolates belonging to the same species. In addition, the nonpathogenic isolates were recovered from asymptomatic tissues, which indicates that they are common endophytic fungi in olive.

Pathogenicity of *Colletotrichum godetiae*, the main causal agent of olive anthracnose in Andalusia (Moral et al. 2014), was also evaluated on lignified tissues. This isolate was included to test the hypothesis that the syndrome of dead branches is caused by phytotoxins produced by this pathogen and often present in rotten fruit (Moral et al. 2009). The pathogenicity test showed that *C. godetiae* was not pathogenic to olive branches. The results support the idea that the isolates of the complex species *C. acutatum* do not cause the typical dieback symptoms through direct infection on lignified tissues, because the toxin produced by the pathogen is the main cause in inducing twig-branch dieback (Moral et al. 2009). However, a wide collection of *Colletotrichum* spp. should be tested for pathogenicity to elucidate the role of their toxins in branch dieback of olive.

Disease resistance of olive cultivars offer an economically sound alternative to chemical control, with minimal environmental impact, which can be integrated in pest management strategies. Here, we conducted the first comparison of cultivar resistance to *N. mediterraneum* causing cankers on olive worldwide, and to *B. dothidea* causing fruit rot of olive in Spain. Trials conducted on detached branches showed that 'Gordal Sevillana' was the cultivar most susceptible to

Table 4. Morphological characteristics of conidia and pycnidia of *Colletotrichum*, *Cytospora*, *Diaporthe*, *Neofusicoccum* and *Phoma*-like isolates obtained from olive

Species	Isolate	Conidia				Pycnidia		
		L × W (μm) ^v	L/W	Morphology	Color	Morphology	Color	Diameter (μm)
<i>Colletotrichum godetiae</i>	CH-21 ^w	(13.05–) 14.35 (–15.65) × (4.03–) 4.59 (–5.15)	3.17 ± 0.48	Aseptate, oblong-fusiform and ligulated	Hyaline	–	–	–
<i>Cytospora</i> sp.	CH-13	(4.92–) 5.47 (–6.01) × (1.30–) 1.61 (–1.92)	3.54 ± 0.32	Aseptate, alantoid form	Hyaline	Erumpent, ostiolate, tuberculate, globous and velvety	Dark	278.5–745.2
<i>Diaporthe</i> sp.	CH-01 ^x	(6.65–) 7.68 (–8.71) × (1.78–) 2.21 (–2.64)	3.60 ± 0.83	Oblong, slightly fusiform and ligulated	Hyaline	Ostiolate, globose, erumpent and velvety	Dark	819.5–1241.1
	CH-03 ^x	(19.12–) 22.50 (–25.88) × (5.27–) 6.26 (–7.25) × (1.91–) 2.40 (–2.89)	–	Filiform and curved	Hyaline	–	–	–
<i>Neofusicoccum mediterraneum</i>	CH-06	(16.30–) 20.18 (–24.06) × (4.93–) 5.86 (–6.79)	2.73 ± 0.80	Oblong, slightly fusiform and ligulated	Hyaline	Ostiolate, globose, erumpent and velvety	Dark	654.5–1778.9
	BOO071	(9.30–) 16.80 (–24.30) × (23.27–) 25.77 (–28.27) × (6.37–) 7.35 (–8.33)	–	Filiform and curved	Hyaline	–	–	–
<i>Phoma</i> -like	CH-04 ^y	(16.30–) 20.18 (–24.06) × (4.93–) 5.86 (–6.79)	3.54 ± 0.93	Fusiform to oblong-fusiform, 0–2 septa, usual truncated base and thin walled	Hyaline	Ostiolate, globose, erumpent and velvety.	Dark	401.9–1685.1
	CH-12	(2.27–) 25.77 (–28.27) × (6.37–) 7.35 (–8.33)	3.57 ± 0.60	Aseptate and fusiform	Hyaline	Ostiolate, globose, erumpent, with conidia in cirrus	Dark	431.2–1465.3
	CH-14	(4.22–) 4.93 (–5.64) × (3.17–) 2.70 (–3.17)	1.87 ± 0.47	Soft texture ellipsoid or ovoid, slightly flattened and thin-walled	Dark	Branched, wooded, velvety, sprawled	Dark	–
	CH-15 ^z	–	–	–	–	Ostiolate, globose	Dark	22.8–73.8
	CH-16	(2.65–) 3.0 (–3.35) × (0.92–) 1.17 (–1.42)	2.66 ± 0.56	Aseptate, oblong to oblong-fusiform	Hyaline	Ostiolate, globose	Dark	60.7–168.5
		(4.23–) 4.69 (–5.15) × (1.15–) 1.78 (–2.06)	2.69 ± 0.48	Aseptate, oblong and thin-walled	Hyaline	Ostiolate, globose	Dark	26.1–142.1
		(2.73–) 3.20 (–3.67) × (0.71–) 0.83 (–0.95)	2.01 ± 0.25	Aseptate and oblong	Hyaline	Ostiolate, globose	Dark	27.7–83.8

^v Mean and range values: length (L) by width (W) (μm). Extremes of the conidial measurements are shown inside parenthesis.

^w This isolate produces conidia in acervuli instead of in pycnidia.

^x Isolates with both conidia types (α and β).

^y Isolate with irregular pycnidia.

^z Conidia were not observed for this isolate.

N. mediterraneum, followed by ‘Santa Caterina’ and ‘San Agostino’, whereas ‘Manzanilla Cacereña’ was the most resistant, followed by ‘Verdial de Huévar’ and ‘Morona’. Detached branches could be under great stress and does not behave physiologically in the same way as

a branch attached to a tree. Thus, results obtained from detached branches could not be absolutely representative of cultivar resistance. In this way, cultivar resistance was also evaluated by using potted plants. In this case, inverted results than those obtained by using

Fig. 2. Phylogenetic analysis of taxa for the combined alignment of internal transcribed spacer and β -tubulin sequences. The evolutionary history was inferred using the neighbor-joining method (Saitou and Nei 1987). The optimal tree with the sum of branch length = 2.64571884 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (2,000 replicates) are shown next to the branches (Felsenstein 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura two-parameter method (Kimura 1980) and are in the units of the number of base substitutions per site. The rate variation among sites was modeled with a γ distribution (shape parameter = 1). The analysis involved 18 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 667 positions in the final data set. Evolutionary analyses were conducted in MEGA6 (Tamura et al. 2013).

Fig. 3. Phylogenetic analysis of taxa for large subunit nuclear ribosomal DNA sequences. The evolutionary history was inferred using the neighbor-joining method (Saitou and Nei 1987). The optimal tree with the sum of branch length = 0.82306726 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (2,000 replicates) are shown next to the branches (Felsenstein 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura two-parameter method (Kimura 1980) and are in the units of the number of base substitutions per site. The rate variation among sites was modeled with a γ distribution (shape parameter = 1). The analysis involved 17 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 782 positions in the final data set. Evolutionary analyses were conducted in MEGA6 (Tamura et al. 2013).

Fig. 4. Disease severity on detached branches and olive plants (Gordal Sevillana) 2 weeks and 1 month, respectively, after inoculation with *Neofusicoccum mediterraneum* isolates BOO071 and CH-06, *Cytospora* sp. isolate CH-13, and *Comoclathris incompta* isolate CH-16. For each isolate, mean values with the same letter are not significantly different according to Tukey's honestly significant difference test ($P < 0.05$).

detached branches were observed on potted plants, where 'Manzanilla Cacereña' was the most susceptible cultivar to *N. mediterraneum*, followed by 'Gordal Sevillana'. 'Verdial de Huévar' was the most resistant cultivar. The last results are in concordance with those obtained by Romero (2012), who indicated that 'Gordal Sevillana' was more susceptible than 'Picual' when live plants were inoculated with *N. mediterraneum*. Concerning *B. dothidea*, results revealed that 'Aloréña de Atarfe' was the most susceptible cultivar, whereas 'Hojiblanca' and 'San Agostino' were the most resistant. These results complement those obtained by Latinović et al. (2013), who described highly resistant native 'Crnjaka' and 'Gloginja' olive. It is interesting to note that the resistance of green olive fruit to *B. dothidea* is not correlated with the resistance of ripe fruit to *C. godetiae*, showing that different resistance mechanisms are at work in both types of fruit (Moral et al. 2014).

Cultivar resistance to Botryosphaeriaceae species affecting lignified tissues of perennial crops has also been studied in mango (*Mangifera indica* L.) and pistachio (*Pistachio vera* L.) (Parfitt et al. 2003). According to our results, 'Spanish Hojiblanca', 'Manzanilla Cacereña', and 'Verdial de Huévar' olive showed a good level of resistance to branch dieback, making cultivar resistance a potential alternative tool against both branch dieback types.

This study has resulted in significant information regarding the etiology of branch dieback of olive as well as cultivar resistance to *N. mediterraneum* and *B. dothidea*. The variation found among fungal species recovered from affected olive trees with cankers and branch dieback symptoms should be taken into account to develop optimum control strategies. Therefore, further research is needed to elucidate the role of many fungal species associated with branch dieback of olive, as well as the environmental and cultural practices that influence the disease.

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