Botryosphaeria and Related Taxa Causing Oak Canker in Southwestern Spain

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ABSTRACT

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Although root disease caused by *Phytophthora cinnamomi* is considered to be the most frequent and damaging disease associated with *Quercus* spp. decline in southwestern Spain, cankers commonly are observed on branches of declining Mediterranean *Quercus* spp. in this region. In surveys carried out in eight declining *Quercus* forests, strips of necrotic inner bark were common on diseased branches. *Botryosphaeria stevensii*, *B. dothidea*, and *Diplodia sarmentorum* consistently were isolated from these branches. Isolates of all three species caused cankers when inoculated onto excised *Quercus* branches. Inoculations on healthy branches in the field also induced canker development, but only *B. stevensii* caused lesions that girdled and killed the branches. The optimum temperature for in vitro growth of *B. stevensii* and *B. dothidea* was above 25°C, with slow growth at 35°C. In contrast, *D. sarmentorum* had an optimum temperature for growth of about 21°C, and did not grow at 35°C. The common occurrence and wide distribution of these pathogens, their association with cankers, and their ability to infect *Quercus* spp. suggest that they may contribute to the *Quercus* spp. decline in southwestern Spain.

Additional keywords: *Botryosphaeria ribis*, *Diplodia mutila*, *Fusicoccum aesculi*, *Othia spiraeae*, *Quercus ilex*, *Quercus suber*

Quercus spp. woodlands, the main climax vegetation in much of southern Spain, are characterized by their capability to protect and create soil under the semiarid climatic conditions of this region. The ecosystems dominated by Mediterranean *Quercus* spp. support a varied and rich wildlife and are important to the rural economy, especially for cattle grazing and cork production (22). For cork production, cork oak trees (*Quercus suber*) are first stripped of about 30 kg of cork by hand when they are about 30 years old. Once harvested, a tree will take between 9 and 10 years to recover its bark, after which time the bark is stripped again. This process can usually be repeated about 10 to 12 times.

Since the beginning of the twentieth century, *Quercus* spp. throughout Europe

have suffered several periods of decline and mortality. In the Mediterranean Basin, a severe decline of *Quercus* spp. has been recognized since the early 1980s. The most affected species in Spain are *Q. suber* and *Q. ilex* and, to a lesser extent, *Q. faginea* and *Q. pyrenaica* (24). A similar decline of *Q. suber* has been reported in Tunisia, Portugal, and Morocco (3,4,10). In Italy, *Q. cerris*, *Q. frainetto*, and *Q. pubescens* are the main species affected (34).

A wide range of factors has been associated with *Quercus* spp. decline, including recurrent periods of severe drought, soil waterlogging, changes in traditional uses of forests, attacks by boring insects, and cankers caused by fungi such as *Biscogniauxia mediterranea* (synonym *Hypoxylon mediterraneum*) and *Botryosphaeria stevensii* (anamorph *Diplodia mutila*) (17–19,23,32,34,36,51). In addition, a bacterial agent, *Brenneria quercina* (synonym *Erwinia quercina*), has been associated with *Quercus* spp. decline in Spain (1).

Quercus canker caused by *Botryosphaeria stevensii* has been described as the most frequent disease associated with *Quercus* spp. decline in Italy (32–34), Morocco (10), and northern Spain. Indeed, trunk cankering caused by *B. stevensii* is considered to be the most common disease causing death of *Q. suber* in northeastern Spain (18,19). In contrast, root disease caused by *Phytophthora cinnamomi* has been regarded as the principal cause of *Quercus*

spp. mortality in southern Spain (3,13,35,40,50). *P. cinnamomi* is responsible for the presence of dead and dying trees in large groups or foci in association with topographical depressions, areas affected by seasonal flooding, and in disturbed zones, such as road margins, fire breaks, and areas supporting heavy grazing by cattle (3,35,50). Charcoal canker, caused by *Biscogniauxia mediterranea*, is well known on Mediterranean *Quercus* spp., mainly *Q. suber*, in southern Spain (48), and has been associated with *Quercus* spp. decline in this region (26,36). There are, however, no reports of other canker fungi associated with *Quercus* spp. decline in southern Spain.

Field observations carried out since 1998 in southwestern Spain (38,40) have revealed that declining *Quercus* forests are affected by a canker disease that differs from charcoal canker caused by *B. mediterranea*. This disease does not always appear in association with *Phytophthora* root disease (40) and it is also present in nondeclining *Quercus* forests (38). Therefore, the aims of the present work were to (i) describe the canker disease present in natural and managed (“dehesas”) *Quercus* communities in southwestern Spain, (ii) identify the causal agent of the disease, and (iii) determine optimum temperatures for growth and pathogenicity of fungi consistently isolated from cankers. A preliminary report has been published (37).

MATERIALS AND METHODS

Field observations and fungal isolations. Surveys were carried out in eight Mediterranean *Quercus* sites located in southwestern Spain and affected by *Quercus* spp. decline (Fig. 1). The predominant *Quercus* spp. were *Q. ilex* subsp. *ballota* or *Q. suber*. A 4-ha plot that included a focus of dead and dying trees was selected at each site. Each plot was surveyed twice: once in spring (April to May) and again in autumn (November to December) 2000. Symptoms and locations of the plots were recorded.

In the spring survey, four affected *Quercus* trees (*Q. ilex* subsp. *ballota* or *Q. suber*, depending on the site) were sampled at each site (Fig. 1). Three cankered but still living branches were removed from each tree. In the autumn survey, 12 trees per site were sampled, and four cankered branches were removed from each tree. For

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both surveys, sampled branches ranged from 25 to 40 cm in length and 1.5 to 3.5 cm in diameter at the middle. The cankers were surface disinfested with ethanol, and the superficial bark tissue was removed with a sterile scalpel. Six chips taken from the necrotic inner bark of each canker were placed on potato dextrose agar (9) acidified with lactic acid (APDA) to suppress bacterial growth (39). The plates were incubated at 22°C in darkness for 2 days followed by another 4 days under 12 h of light and 12 h of dark at 22°C. Fungi growing from the chips were transferred to fresh plates of APDA. Representative strains of all three species have been deposited at the Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands as CBS 1125545-112547 (*Botryosphaeria stevensii*), CBS 113190-113191 (*B. dothidea*), and CBS 113188-113189 (*D. sarmentorum*). Other strains are available from the authors.

Characterization of isolates. Identifications were based partly on cultural characteristics, but principally on micromorphology on the host and in culture. Sporulation on APDA was stimulated by incubation under fluorescent light (12 h day⁻¹) at 25°C. Conidiomata and conidia from pure cultures were mounted in 0.005% acid fuchsin in lactophenol. Lengths and widths of 50 conidia from three different isolates of each of three morphological groups obtained from *Q. ilex* subsp. *ballota*, and three other isolates of each of three mor-

phological groups obtained from *Q. suber*, were measured at a magnification of ×1,000. Ascomata were taken directly from infected bark, mounted, stained, and observed with the microscope. Size and shape of the ascomata, 15 asci, and 30 ascospores were recorded.

Mycelial growth rates of the same six isolates from each of the three morphological groups were determined on APDA. A 7-mm-diameter plug taken from the colony margin was placed at the center of a 9-cm-diameter petri dish, and three plates of each isolate were incubated separately at 5, 10, 15, 20, 25, 30, or 35°C in the dark. Two colony diameters per plate were measured after 2 days and again after 4 days, and the diameter of the inoculum plug subtracted. Diameter growth rates were calculated as millimeters per day, using data from 2- and 4-day measurements. The experiment was conducted twice. Regression curves were fitted to the values of daily growth rate versus temperature using Statistix for Windows software (Analytical Software, Tallahassee, TN), and cardinal temperatures for growth were calculated.

Pathogenicity tests. Two isolates, one obtained from *Q. ilex* subsp. *ballota* and one from *Q. suber*, were chosen from each of the three morphological groups to determine their pathogenicity under laboratory and under field conditions.

Experiment 1: Inoculation of excised branches. For inoculation in the laboratory,

segments of branches 16 to 32 cm long and 6 to 15 mm in diameter were taken from mature and symptomless *Q. ilex* subsp. *ballota* trees at a site not affected by decline in Córdoba, southern Spain. The branch segments were sealed at both ends with Parafilm to reduce desiccation, and the bark surface was disinfested with ethanol at the inoculation point situated in the middle of the branch. Holes 7 mm in diameter were made in the bark with a cork borer to remove the outer bark but leave the inner bark intact. A 7-mm-diameter inoculum plug taken from an APDA plate then was placed in each hole and covered with parafilm. In the controls, sterile APDA plugs were used in place of fungal mycelium. The branches were incubated at 15, 20, 25, or 30°C in moist chambers with 12 h of light and 12 h of darkness per day. Six replicate branches were taken from different trees for each isolate and temperature tested. Lesion lengths were measured after 4 weeks. The outer bark was removed along the entire length of the lesions to observe discoloration (necrosis). Data were tested for normality by Bartlett's test. Because this test showed equality of variances, analysis of variance (ANOVA) and calculation of Fisher's protected least significant difference (45) were performed using Statistix for Windows, with data from control branches omitted (lesions = 0 mm).

Experiment 2: Field inoculations. Another inoculation experiment was performed on symptomless, mature *Q. ilex* subsp. *ballota* trees located in the same nondeclining plot where the branches for experiment 1 were collected. There were two trials, one in December 2000 (winter inoculation), and another in March 2001 (spring inoculation). The method described above was used to inoculate branches 6 to 15 mm diameter. Each treatment was assigned to a different branch on a tree (block) in a randomized complete block design, with six replicate trees. Statistical analysis was the same as described for experiment 1, except for the experimental design used.

After each experiment, inoculated and control branches were removed. To determine the distribution of fungi along the cankers, isolations were made by taking 2- to 3-mm² chips of inner bark, under aseptic conditions, at 1-cm intervals from the inoculation point in both the proximal and distal directions. The bark chips were plated on APDA and incubated at 22°C in the dark for 2 days, followed by another 15 to 30 days under 12 h of light and 12 h of dark to encourage sporulation.

RESULTS

Symptoms. In spring 2000, branch die-back associated with bark cankering was detected at four of the eight decline sites surveyed (Table 1). In contrast, trees at all the sites surveyed in autumn had cankers

Fig. 1. Locations of forest sites surveyed in southwestern Spain for Mediterranean *Quercus* spp. canker disease. 1: La Rozuela, 2: Viñuela Alta, 3: Los Labrados, 4: Las Navas y el Berrocal, 5: La Encarnación, 6: La Pizarra, 7: Monte Mogeia Luenga, 8: San Carlos del Tiradero.

and yellowing, browning, and wilting of leaves on scattered branches. All branches with wilted leaves attached also had girdling cankers that were more easily visible when the outer bark was removed. However, not all defoliated, dead branches exhibited cankers. Trunk cankers were frequent in *Q. suber*. These cankers were elongated, corky swellings, 5 to 20 cm long, frequently with a central crack exposing the necrotic inner bark. Girdling cankers were not observed on *Q. suber* stems. Incidence of other detected diseases and insect pests are summarized in Table 2.

Characteristics of the pathogens. A high proportion of the canker chips yielded fungal colonies on APDA (Table 1). Initially, all colonies were white, becoming gray-green to gray-black. After 15 days at 22°C, three different colony morphologies were apparent. In only three plots (plots 1, 2, and 3) were all three morphological groups isolated (Table 1). From a total of 112 isolates obtained in pure culture, 47 were gray with abundant aerial mycelium and were identified as *B. stevensii*, another 54 isolates were dark gray with sparse aerial mycelium (*D. sarmentorum* isolates), and the remaining 11 isolates were dark gray with abundant aerial mycelium (*B. dothidea* isolates). Although identification was based on pycnidia formed in pure cultures, pycnidia also were observed in

diseased cortical tissues. Micromorphological characteristics of six isolates selected from each of the above colony morphology groups distinguished the three different species.

Conidiophores were absent in the *B. stevensii* isolates. Conidiogenous cells measured 17 by 26 by 4 µm and were hyaline, smooth, cylindrical, aseptate, and holoblastic, producing a single conidium at the tip but later proliferating percurrently, giving rise to two to three indistinct annellations, or proliferating at the same position, resulting in periclinal thickenings. Conidia from cultures were identical to those from stromata on diseased tissue. They were hyaline and aseptate with a smooth, relatively thick wall. Approximately 80% were cylindrical, with a rounded apex and a rounded or truncate base. The average dimensions and standard deviations of the cylindrical conidia were 28 ± 3 by 13 ± 1.5 µm, with a length:width ratio of 2.1 ± 0.3. The remaining 20% were subglobose and averaged 16.5 ± 3 by 13.5 ± 1 µm, with a length:width ratio of 1.2 ± 0.2. Cylindrical, pale-brown, and one-septate conidia were seen occasionally. Hyaline spermatia also were produced in pure cultures and on infected tissues, and these were 2 by 1 µm. They were produced intermixed with conidia or in separate locules. These isolates conform to *D. mutila*, the anamorph of *B. stevensii* (28,42).

Conidiophores also were absent in the *D. sarmentorum* isolates. Conidiogenous cells were hyaline, smooth, cylindrical, aseptate, holoblastic at first, later proliferating percurrently with one or two indistinct annellations, arising from the inner wall of the pycnidium, measuring 12 to 15 (or 17) by 3 to 4.5 µm. Conidia from cultures were identical to those from diseased tissue and initially were thick-walled, hyaline, and aseptate, becoming dark brown and medianly one-septate, externally smooth, internally ornamented and verruculose, straight, ovoid to oblong, apex obtuse, base truncate, 24 ± 2 by 9.5 ± 1 µm, with a length:width ratio of 2.6 ± 0.3. Although these isolates have certain characters that could place them within Sutton's (47) concept of *D. mutila*, they conform more closely with *D. sarmentorum*, which is thought to be the anamorph of *Othia spiraeae* (2).

In the *B. dothidea* isolates, the conidiophores were hyaline, cylindrical, smooth, and sometimes branched. Conidiogenous cells were hyaline, smooth, thin-walled, and cylindrical, up to 28 by 2 to 2.5 µm, holoblastic, occasionally proliferating at the same level and resulting in periclinal thickenings. Conidia from cultures were identical to those from diseased bark: hyaline, thin-walled, smooth, fusiform to narrowly ellipsoidal, the contents granular, apex subacute, base truncate with a minute

Table 1. Fungal isolations from cankered branches of Mediterranean *Quercus* spp. in southwestern Spain^y

Plot	Species ^z	<i>Botryosphaeria stevensii</i>				<i>Diplodia sarmentorum</i>				<i>Botryosphaeria dothidea</i>			
		Spring		Autumn		Spring		Autumn		Spring		Autumn	
		Inc	Isol	Inc	Isol	Inc	Isol	Inc	Isol	Inc	Isol	Inc	Isol
1	<i>Qi</i>	0	0	12.5	83.3	75	73.8	16.6	54.1	8.3	45	4.2	49.9
2	<i>Qi</i>	0	0	27.7	65	0	0	16.6	52.7	100	28	0	0
3	<i>Qs</i>	...	...	4.2	58.3	...	...	6.3	66.6	...	...	2.1	100
4	<i>Qi</i>	...	...	0	0	...	...	8.3	41.6	...	...	4.2	66.6
5	<i>Qi</i>	...	...	50	75	...	...	0	0	...	...	0	0
6	<i>Qi</i>	33.3	81.5	20	37.5	41.6	28.3	10	33.3	0	0	0	0
7	<i>Qs</i>	100	63.5	ns	ns	0	0	ns	ns	0	0	ns	ns
8	<i>Qs</i>	...	...	ns	ns	...	...	ns	ns	...	...	ns	ns

^y Inc = incidence, percentage of affected branches with positive fungal isolation; Isol = isolation, percentage of necrotic bark chips yielding a fungal colony when plated on potato dextrose agar acidified with lactic acid; ... = cankered branches not present; ns = not sampled.

^z Tree species: *Qi* = *Quercus ilex* subsp. *ballota* and *Qs* = *Quercus suber*.

Table 2. Diseases and insect pests detected at the experimental plots

Plot	Species ^y	Forest type	Main disease	Other diseases	Insect pests
1	<i>Qi</i>	Dehesa ^z	Phytophthora root rot (<i>Phytophthora cinnamomi</i>)	Botryosphaeria canker, Charcoal canker (<i>Biscogniauxia mediterranea</i>), Bacterial canker (<i>Brenneria quercina</i>), Witches' brooms (<i>Taphrina kruchii</i>)	<i>Cerambyx</i> sp.
2	<i>Qi</i>	Dehesa	Phytophthora root rot	Botryosphaeria canker, Charcoal canker	<i>Cerambyx</i> sp., <i>Coroebus undatus</i>
3	<i>Qs</i>	Dehesa	Botryosphaeria canker	Witches' brooms, Scab (<i>Spilocaea quercus-ilex</i>)	<i>Cerambyx</i> sp.
4	<i>Qi</i>	Dehesa	Botryosphaeria canker	Charcoal canker, Bacterial canker	<i>Coroebus undatus</i>
5	<i>Qi</i>	Natural	Phytophthora root rot	Botryosphaeria canker	<i>Cerambyx</i> sp., <i>Kermococcus</i> sp.
6	<i>Qi</i>	Dehesa	Phytophthora root rot	Botryosphaeria canker, Scab	...
7	<i>Qs</i>	Natural	Phytophthora root rot	Botryosphaeria canker, Bacterial canker	<i>Coroebus undatus</i> , <i>Platypus cylindrus</i>
8	<i>Qs</i>	Natural	Phytophthora root rot	Botryosphaeria canker, Bacterial canker	...

^y Tree species: *Qi* = *Quercus ilex* subsp. *ballota* and *Qs* = *Quercus suber*.

^z Dehesa: Forest managed for cattle grazing, maintained with less than 30 trees/ha.

marginal frill, widest in the upper third of the conidium, mainly aseptate but occasionally one-septate. Average dimensions were 22.5 ± 3.5 by 4.5 ± 1 μm , with a length:width ratio of 5.2 ± 1.1 . Characters of this isolates on the host and in culture

conformed to the *Fusicoccum* anamorphs of the genus *Botryosphaeria* (7,27). They fit well within the description of the type species *Fusicoccum aesculi* as emended by Crous and Palm (7), which is the anamorph of *B. dothidea* (27).

Ascomata were seen only immersed in the diseased bark of trunk cankers on *Q. suber* trees that were stripped of their bark 2 years before sampling. They appeared as dark-brown to black, multilocular structures, containing bitunicate, hyaline, clavate asci, 112 ± 9.5 by 25 ± 2 μm . The eight ovoid ascospores were mainly hyaline, smooth, and aseptate, but brown and two-septate ascospores rarely were seen in the same ascus with hyaline, aseptate ascospores. Ascospores were 28.5 ± 2 by 13 ± 1.5 μm , with a length:width ratio of 2.2 ± 0.3 . These characteristics fit closely with the description of *B. stevensii* (29,42). No other teleomorph was observed in infected branches or in trunk cankers, and none formed in culture. A total of 58 monoascosporic cultures were obtained by the methods developed by Trapero and Kaiser (49). Morphology of these cultures, including the pycnidia and conidia, on APDA was identical to the isolates obtained from necrotic bark and identified as *B. stevensii*.

Growth rates were plotted against temperature (Fig. 2), and the relationship was described by a fourth degree polynomial. ANOVA and Fisher's test indicated significant differences in average growth rate at the different temperatures tested, but growth rates of isolates of the same species did not differ significantly from one another at each temperature. The estimated optimum temperature for growth of *D. sarmentorum* was near 22°C but was higher ($>25^\circ\text{C}$) for *B. stevensii* and *B. dothidea*. In addition, *B. stevensii* and *B. dothidea* exhibited slow growth at 35°C , but *D. sarmentorum* did not grow at this temperature.

Pathogenicity tests. All inoculations with fungal isolates resulted in lesions of the inner bark. In experiment 2, wilting of the foliage and branch death also occurred. No lesions were observed in control branches. There was no difference between proximal and distal lesion extension from the inoculation point in either inoculation trial. In all experiments, the inoculated species was reisolated from all the lesions. As found with natural cankers, the pathogen could be isolated from the entire length of the canker. An unidentified *Fusarium* sp. and bacteria occasionally were isolated from the lesions.

Experiment 1: Excised branches. Four weeks after inoculation, some of the bark lesions almost reached the end of the branches (16 to 32 cm). Mean lesion lengths are shown in Table 3. The ANOVA indicated that average lesion length differed significantly depending on the isolate inoculated, incubation temperature, and their interaction. Individual ANOVA for each temperature tested and comparison of means (Table 3) showed significant differences among isolates at 15 and 20°C . At these temperatures, both isolates of *B. stevensii* produced significantly longer

Fig. 2. Temperature-growth relationships for **A**, *Botryosphaeria stevensii*, **B**, *Diplodia sarmentorum*, and **C**, *B. dothidea*. Arrows indicate the estimated optimum growth temperature. Each data point is the observed average growth rate of one of six isolates.

lesions than all the other fungi, although, at 20°C, they did not differ from isolate DSE27 of *D. sarmentorum*. In contrast, differences in lesion length were smaller at 25°C and no significant differences could be detected among species at 30°C.

Experiment 2: Field trees. Six months after the winter inoculations, small lesions developed on the inoculated branches. Branches inoculated with *D. sarmentorum* and *B. dothidea* showed lesions surrounded by callus, but branch girdling was not observed. In contrast, the lesions that developed on branches inoculated with *B. stevensii* did not show callus production. In these branches, foliar symptoms were not evident, even on two girdled branches. ANOVA and comparison of means (Table 4) showed that lesions were significantly longer in branches inoculated with the isolate BSE1 of *B. stevensii*, obtained from *Q. ilex* subsp. *ballota*, than with the other isolates.

Three months after the spring inoculations, foliar wilting, girdling, and branch death were evident in branches inoculated with both isolates of *B. stevensii*. However, in branches inoculated with *D. sarmentorum* or *B. dothidea*, the bark lesions did not girdle the branches, and no foliar symptoms developed. Callus production was not detected in the spring inoculations with any of the fungal species. The ANOVA revealed that average lesion length differed significantly depending on the isolate inoculated, season of inoculation (winter or spring), and their interaction. Individual ANOVA for inoculation seasons and comparison of means (Table 4) showed that lesions were significantly longer on branches inoculated with both *B. stevensii* isolates than in branches inoculated with isolates of the other two species. In addition, isolate BSE1 of *B. stevensii*, obtained from *Q. ilex* subsp. *ballota*, produced lesions significantly longer than isolate BSA1, which was isolated from *Q. suber*.

DISCUSSION

Field surveys carried out since 1998 on natural forests and managed forests ("dehesas") of Mediterranean *Quercus* spp. revealed that trees showing foliar wilting and branch death in southwestern Spain invariably had bark lesions or cankers (38,40). However, defoliated, dead branches without cankers were common in declining *Quercus* forests. In six of the eight plots surveyed in this work, trees were affected by root disease caused by *P. cinnamomi* (40), which also can induce defoliation and branch dieback.

Three different *Botryosphaeria* or related species consistently were isolated from the necrotic bark. Some of the characteristics used for identification, such as color and septation of conidia or stroma morphology, are plastic and affected by age of colony (8,15). Five species of *Diplodia* have been reported on *Quercus* spp.,

namely, *D. longispora*, *D. mutila*, *D. quercina*, *D. sarmentorum*, and *D. suberina* (11,12,42). The anamorphic genus *Diplodia* includes approximately 1,000 species and is poorly defined (8,14), and these fungi normally are referred to by their holomorph names.

In this study, the species most frequently isolated was identified as *B. stevensii*. The sexual state was identified in 2000 from trunk cankers on cork oaks stripped of their bark in 1998. Certain anamorph features of the isolates we studied coincide with *B. quercuum*. For example, subglobose conidia are considered to be more typical of *B. quercuum* than of *B. stevensii*. The main character that distinguishes these two species (42) is the length:width ratio of conidia. Both subglobose and cylindrical conidia were seen in the strains studied here. Length:width ratios of the subglobose conidia were 1.2, which agrees with the value (1.5) for *B. quercuum*, whereas the length:width ratios of the cylindrical conidia averaged 2.1, more typical of *B. stevensii* (42). Phylogenetic relationships among *Botryosphaeria* spp. and related taxa based on rDNA sequence data suggest that the name *B. stevensii* may have been applied to more than one species and that isolates named as *B. quercuum* also might be called *B. stevensii* (53).

The second most common species consistently associated with *Quercus* cankers in southwestern Spain was identified as *D. sarmentorum* (46), which has been regarded as the anamorph of *O. spiraeae* (2,43). As pointed out by Laundon (16), *D. sarmentorum* clearly is related to the anamorphs of *Botryosphaeria* spp. Denman et

al. (8) suggested that the genus *Othia* should be synonymized with genus *Botryosphaeria*, but further morphological and molecular studies were needed. The teleomorph of *D. sarmentorum* was not seen in the present study.

Morphology of the third of the species from southwestern Spain conforms to the *Fusicoccum* anamorphs of *Botryosphaeria* spp. (7,8,15,27,47). These isolates closely resemble the type species *F. aesculi*, which is the anamorph of *B. dothidea* (7). They also resemble the *Fusicoccum* anamorph of *B. ribis* (21,31), although the conidia of our isolates were narrower than those described for *B. ribis* on some other hosts (25,31,39,47). It was not possible to obtain the teleomorph in our cultures, nor was it seen on naturally infected material from the field. Although *B. dothidea* and *B. ribis* have been considered synonyms (6,21,41,43), they also have been treated as separate species (11,52). Phylogenetic analyses based on rDNA internal transcribed spacer sequence data support the synonymy of the two species (7,8,15,27,30) or their distinction (44,53).

Growth-temperature relationships for our *B. dothidea* isolates are in agreement with data obtained for isolates from gum cistus (39), which grew slowly at 10 and 35°C, with optimum temperature for growth of about 28°C. However, isolates from other hosts did not grow at 35°C (5,15). Our *B. stevensii* isolates grew at 35°C, in contrast to the observations of Jacobs and Rehner (15). In our tests, only *D. sarmentorum* isolates failed to grow at 35°C. Inoculation of excised branches indicated that all three species were able to

Table 3. Mean lesion length (cm) on excised branches of *Quercus ilex* subsp. *ballota* inoculated with *Botryosphaeria stevensii*, *B. dothidea*, and *Diplodia sarmentorum* and incubated at different temperatures^z

Fungal species	Isolates	Incubation temperature (°C)			
		15	20	25	30
<i>B. stevensii</i>	BSA1	20.3 a	18.3 a	18.1 ab	17.5 a
	BSE1	18.8 ab	18.0 a	19.1 a	17.9 a
<i>D. sarmentorum</i>	DSE27	13.3 bc	15.4 ab	19.9 a	14.4 a
	DSE14	9.3 cd	9.8 bc	14.9 ab	15.8 a
<i>B. dothidea</i>	BDE2	5.9 de	8.1 c	12.3 b	16.7 a
	BDE5	1.6 e	5.0 c	19.8 a	13.3 a

^z For each column, values with the same letter do not differ significantly according to Fisher's least significant difference test ($P = 0.05$).

Table 4. Mean lesion length (cm) on branches of *Quercus ilex* subsp. *ballota* inoculated in the field with *Botryosphaeria stevensii*, *B. dothidea*, and *Diplodia sarmentorum*^z

Fungal species	Isolates	Lesion length (cm)	
		Winter inoculation	Spring inoculation
<i>B. stevensii</i>	BSE1	11.4 a	36.3 a
	BSA1	6.4 b	21.2 b
<i>D. sarmentorum</i>	DSE14	2.2 b	2.8 c
	DSE27	2.1 b	3.4 c
<i>B. dothidea</i>	BDE2	2.5 b	2.5 c
	BDE5	2.0 b	3.3 c

^z For each column, values with the same letter do not differ significantly according to Fisher's least significant difference test ($P = 0.05$).

infect and survive in weakened cortical tissue at over 25°C. Differences in aggressiveness among species and isolates could be detected at lower temperatures.

Experimental inoculation of living branches of healthy trees suggests that there is no requirement that the host be weakened for cankers to develop. Canker development by *B. stevensii* isolates was clearly favored by spring conditions over winter conditions. Luque et al. (20) showed that lesion lengths on seedlings of *Q. suber* inoculated with *B. stevensii* were directly related to the minimum temperature at the time of inoculation; the largest lesions developed on seedlings inoculated at the hottest time of the year (August). In our experiments, only the spring inoculation with *B. stevensii* isolates produced foliar wilting, girdling of branches, and their ultimate death.

The results of our study indicate that *B. stevensii* is a primary pathogen of *Q. ilex* subsp. *ballota* in southwestern Spain, as was described by Luque and Girbal (18) for *Q. suber* in the northeastern part of the country and Frisullo et al. for different *Quercus* spp. in Italy (12). *B. stevensii* has been cited as one of the biotic factors implicated in decline of Mediterranean *Quercus* spp. in northern Spain (19), Italy (34), and Morocco (10). The two other fungi considered in our work were shown to be able to produce extensive bark necrosis on weakened branches, although they did not girdle vigorous living branches, as also was found for Italian isolates of both species (12). Thus, although only *B. stevensii* can be considered a primary pathogen of *Q. ilex* subsp. *ballota*, it is possible that all three species can contribute to the decline of Mediterranean *Quercus* spp. in southwestern Spain.

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LITERATURE CITED

- Biosca, E. G., González, R., López-López, M. J., Soria, S., Monton, C., Pérez-Laorga, E., and López, M. M. 2003. Isolation and characterization of *Brenneria quercina*, causal agent for bark canker and drippy nut of *Quercus* spp. in Spain. *Phytopathology* 35:485-492.
- Booth, C. 1958. Studies of pyrenomycetes. III. *Othia spiraeae* (Fuckel) Fuckel, syn. *Diplodia sarmentorum* (Fr.) Fr. Trans. Br. Mycol. Soc. 41:335-340.
- Brasier, C. M. 1996. *Phytophthora cinnamomi* and oak decline in southern Europe. Environmental constraints including climate change. *Ann. Sci. For.* 53:347-358.
- Brito de Carvalho, J. H. 1993. "Stress" do Sobreiro e da Azinheira ou a Doença de Lopes Pimentel? CIDER, Univ. Algarve, Faro, Portugal.
- Brooks, F. E., and Ferrin, D. M. 1994. Branch dieback of southern California chaparral vegetation caused by *Botryosphaeria dothidea*. *Phytopathology* 84:78-83.
- Cline, W. O. 1994. Infection of cold-injured blueberry stem by *Botryosphaeria dothidea*. *Plant Dis.* 78:1010.
- Crous, P. W., and Palm, M. E. 1999. Reassessment of the anamorph genera *Botryodiplodia*, *Dothiorella* and *Fusicoccum*. *Sydowia* 52:167-175.
- Denman, S., Crous, P. W., Taylor, J. E., Kang, J. C., Pascoe, I., and Wingfield, M. J. 2000. An overview of the taxonomic history of *Botryosphaeria*, and re-evaluation of its anamorphs based on morphology and ITS rDNA phylogeny. *Stud. Mycol.* 45:129-140.
- Dhingra, O. D., and Sinclair, J. B. 1995. Basic Plant Pathology methods. CRC Press, Boca Raton, FL.
- El-Badri, N., and Abadie, M. 2000. Observations on the dynamic of *Diplodia mutila* Fr. apud Mont. development on the cork-oak, *Quercus suber* L., in Morocco. *Cryptogam. Mycol.* 21:235-248.
- Farr, D. F., Bills, G. F., Chamuris, G. P., and Rossman, A. Y. 1989. Fungi on plants and plants products in the United States. American Phytopathological Society Press, St. Paul, MN.
- Frisullo, S., Camele, I., Carlucci, A., and Lops, F. 2000. *Botryosphaeria* and *Botryosphaeria*-like micromycetes on declining oaks in Apulia and Basilicata. *Petria* 10:1-10.
- Gallego, F. J., Pérez de Algaba, A., and Fernández-Escobar, R. 1999. Etiology of oak decline in Spain. *Eur. J. For. Pathol.* 29:17-27.
- Hawksworth, D. L., Kirk, P. M., Sutton, B. C., and Pegler, D. M. 1995. Dictionary of the fungi. CAB International, Wallingford, Oxon, UK.
- Jacobs, K. A., and Rehner, S. A. 1998. Comparison of cultural and morphological characters and ITS sequences in anamorphs of *Botryosphaeria* and related taxa. *Mycologia* 90:601-610.
- Laundon, G. F. 1973. *Botryosphaeria obtusa*, *B. stevensii* and *Othia spiraeae* in New Zealand. *Trans. Br. Mycol. Soc.* 61:369-374.
- Luisi, N., Lerario, P., and Vannini, A., eds. 1993. Recent advances in studies on oak decline. Proc. Int. Congr. Recent Adv. Studies Oak Decline, Brindisi, Italy.
- Luque, J., and Girbal, J. 1989. Dieback of cork oak (*Quercus suber*) in Catalonia (NE Spain) caused by *Botryosphaeria stevensii*. *Eur. J. For. Pathol.* 19:7-13.
- Luque, J., Parladé, J., and Pera, J. 2000. Pathogenicity of fungi isolated from *Quercus suber* in Catalonia (NE Spain). *For. Pathol.* 30:247-263.
- Luque, J., Parladé, J., and Pera, J. 2002. Seasonal changes in susceptibility of *Quercus suber* to *Botryosphaeria stevensii* and *Phytophthora cinnamomi*. *Plant Pathol.* 51:338-345.
- Maas, J. L., and Uecker, F. A. 1984. *Botryosphaeria dothidea* cane canker of thornless blackberry. *Plant Dis.* 68:720-726.
- Montoya, J. M. 1988. Los alcornoques. Manuales Técnicos SEA. MAPA, Madrid.
- Montoya, J. M. 1992. Mortandad de *Quercus*, la perspectiva selvícola y los antecedentes climáticos. *Ecología* 6:123-130.
- Montoya, J. M. 1994. ¿Qué es la "seca de los Quercus"? Actas de la X Reunión Anual del Grupo de Trabajo Fitosanitario de Forestales, Parques y Jardines, Madrid.
- Morgan-Jones, G., and White, J. F. 1987. Notes on the Coelomycetes. II. Concerning the *Fusicoccum* anamorph of *Botryosphaeria ribis*. *Mycotaxon* 30:117-125.
- Navarro, R. M., and Fernández, P. 2000. El síndrome de la seca del encinar. Propuesta de solución para el Valle de los Pedroches. Fundación Ricardo Delgado Vizcaíno, Pozoblanco, Córdoba, Spain.
- Pennycook, S. R., and Samuels, G. J. 1985. *Botryosphaeria* and *Fusicoccum* species associated with ripe fruit rot of *Actinidia deliciosa* (kiwifruit) in New Zealand. *Mycotaxon* 24:445-458.
- Phillips, A. J. L. 2000. Excoriose, cane blight and related diseases of grapevines: a taxonomic review of the pathogens. *Phytopathol. Mediterr.* 39:341-356.
- Phillips, A. J. L. 2002. *Botryosphaeria* species associated with diseases of grapevines in Portugal. *Phytopathol. Mediterr.* 41:3-18.
- Phillips, A. J. L., and Lucas, M. T. 1997. The taxonomic status of *Macrophoma flaccida* and *Macrophoma reniformis* and their relationship to *Botryosphaeria dothidea*. *Sydowia* 49:150-159.
- Punithalingam, E., and Holliday, P. 1973. *Botryosphaeria ribis*. C.M.I. Descriptions of Pathogenic Fungi and Bacteria No. 395. Kew, UK.
- Ragazzi, A., Fedi, I. D., and Mesturino, L. 1989. The oak decline: a new problem in Italy. *Eur. J. For. Pathol.* 19:105-110.
- Ragazzi, A., Moricca, S., and Dellavalle, I. 1999. Water-stress and development of canker by *Diplodia mutila* on *Quercus robur*. *J. Phytopathol.* 147:425-428.
- Ragazzi, A., Moricca, S., Dellavalle, I., and Turco, E. 2000. Decline of oak species in Italy. Problems and perspectives. *Accademia Italiana di Scienze Forestali*, Firenze, Italy.
- Sánchez, M. E., Caetano, P., Ferraz, J., and Traperó, A. 2002. *Phytophthora* disease of *Quercus ilex* in southwestern Spain. *For. Pathol.* 32:5-18.
- Sánchez, M. E., Navarro, R. M., Traperó, A., and Fernández, P. 2000. La "seca" de encinas y alcornoques: una visión histórica. *Montes* 62:29-39.
- Sánchez, M. E., Venegas, J., Romero, M. A., Blanco, M. A., and Traperó, A. 2001. Fungal canker diseases associated with Mediterranean *Quercus* decline in southern Spain. Pages 64-66 in: Proc. 11th Congr. Mediterr. Phytopathol. Union. Évora, Portugal.
- Sánchez, M. E., Venegas, J., Romero, M. A., Phillips, A. J. L., and Traperó, A. El chancro de encinas y alcornoques causado por *Botryosphaeria* spp. en Andalucía. *Boletín Sanidad Veg. Plagas* 29. In press.
- Sánchez-Hernández, M. E., Gutiérrez-García, J., and Traperó-Casas, A. 2002. *Botryosphaeria* canker of *Cistus ladanifer*. *Plant Pathol.* 51:364-372.
- Sánchez-Hernández, M. E., Sánchez Solana, J. E., Navarro Cerrillo, R. M., Fernández Rebollo, P., and Traperó Casas, A. Incidencia de la podredumbre radical causada por *Phytophthora cinnamomi* en masas de *Quercus* en Andalucía. *Boletín Sanidad Veg. Plagas* 29. In press.
- Shahin, E. A., and Clafflin, L. E. 1980. Twig blight of Douglas-fir: A new disease caused by *Dothiorella dothidea*. *Plant Dis.* 64:47-50.
- Shoemaker, R. A. 1964. Conidial states of some *Botryosphaeria* species on *Vitis* and *Quercus*. *Can. J. Bot.* 42:1297-1301.
- Sivanesan, A. 1984. The bitunicate Ascomycetes and their anamorphs. J. Cramer, Vaduz, Germany.
- Smith, D. R., and Stanosz, G. R. 2001. Molecular and morphological differentiation of *Botryosphaeria dothidea* (anamorph *Fusicoccum aesculi*) from some other fungi with *Fusicoccum* anamorphs. *Mycologia* 93:505-515.
- Steel, G. D., and Torrie, J. H. 1985. Biostatística: Principios y procedimientos. McGraw-Hill, Bogotá, Colombia.
- Stevens, N. E. 1936. Two species of *Physalospora* in England. *Mycologia* 28:330-336.
- Sutton, B. C. 1980. The Coelomycetes. Commonwealth Mycological Institute, Kew, UK.
- Torres Juan, J. 1985. El *Hypoxyton mediterraneum* (De Not) Mill y su comportamiento en los encinares y alcornoques andaluces. *Bo-*

- letin Sanidad Veg. Plagas 11:185-191.
49. Trapero, A., and Kaiser, W. J. 1992. Development of *Didymella rabiei*, the teleomorph of *Ascochyta rabiei*, on chickpea straw. Phytopathology 82:1261-1266.
 50. Tuset, J. J., Hinarejos, C., Mira, J. L., and Cobos, J. M. 1996. Implicación de *Phytophthora cinnamomi* Rands en la enfermedad de la seca en encinas y alcornoques. Boletín Sanidad Veg. Plagas 22:491-499.
 51. Vannini, A., Valentini, R., and Luisi, N. 1996. Impact of drought and *Hypoxyton mediterraneum* on oak decline in the Mediterranean region. Ann. Sci. For. 53:753-760.
 52. von Arx, J. A. 1987. Plant Pathogenic Fungi. J. Cramer, Berlin.
 53. Zhou, S. G., and Stanosz, G. R. 2001. Relationships among *Botryosphaeria* species and associated anamorphic fungi inferred from the analyses of ITS and 5.8S rDNA sequences. Mycologia 93:516-527.