Isolation and Characterization of *Brenneria quercina*, Causal Agent for Bark Canker and Drippy Nut of *Quercus* spp. in Spain

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ABSTRACT

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The drippy nut disease of oak was first described in California in 1967 and, since then, the causal agent has not been reported in any other area. This study describes for the first time in Europe the isolation of *Brenneria* (*Erwinia*) *quercina* from bark canker in addition to drippy bud and drippy nut in *Quercus ilex* and *Q. pyrenaica*. The bark canker and drippy bud symptoms were not previously described as caused by this bacterium. No fungal pathogens were associated with any of the symptoms. Physiological and biochemical characterization identified the pathogenic isolates from Spain as belonging to *B. quercina*, similar to the reference strain CFBP 1266. Fatty acid profiles of the Spanish isolates also were similar to the strain of *B. quercina* from California. Serological analysis by indirect immunofluorescence and enzyme-linked immunosorbent assay using polyclonal antisera against the reference strain of *B. quercina* and one Spanish oak isolate revealed some antigenic heterogeneity between isolates of different origins. Pathogenicity tests demonstrated that the Spanish isolates were able to reproduce internal symptoms of necrosis and acorn exudation in *Q. ilex* and *Q. pyrenaica* and suggest that *B. quercina* may be associated, among other causes, with the oak decline syndrome affecting Spanish oak forests.

Additional keywords: inoculation, phenotypic, serology.

In the last few decades, diverse symptomatology associated with oak decline syndrome has been reported in several European countries (4–6,21,26). The syndrome affects several *Quercus* species and, in Spain, it has been described on *Q. ilex* (holm oak) subsp. *ilex* and subsp. *rotundifolia*, *Q. pyrenaica*, *Q. suber* (cork oak), and *Q. faginea*. The affected trees suffer progressive loss of vigor, foliage reduction, and early leaf senescence. Rapid decline and death of the trees has been reported in some isolated cases. Among other symptoms, the affected oak trees often showed small longitudinal lesions of a few centimeters on the trunk bark which produce exudates, especially during the spring and autumn (5,28). In some cases, bark cankers were very noticeable, reaching decimeters in length and affecting development. In other cases, very limited external symptoms were observed and it was only after removing the outer bark that extensive necrotic areas were revealed (28).

Abiotic and biotic factors are considered responsible for oak decline in Europe and the United States acting simultaneously or in succession (1,16,32). Leininger (16) proposed that temperature and drought stress were related to this syndrome, suggesting that an increase of 1°C in the maximum temperature during the growing season could have deleterious long-term effects on oak forests. In Spain, some studies have reached similar conclusions (8). In addition, certain fungi are known to be involved in the oak decline in Europe and California (i.e., *Armillaria* sp. [18], *Phytophthora cinnamomi* and *Phytophthora ramorun* [4,5,19,22,30], *Diplodia* sp. [23], and *Hypoxilon mediterraneum* [31]). The affected oak species were *Q. ilex* and *Q. suber* in Spain and *Lithocarpus densiflorus*, *Q. agrifolia,* and *Q. kelloggi* in California (4,22,30). None of these pathogens can be considered as the universal causal agent of the oak decline syndrome and the relative role of these organisms is still unclear in many cases.

With regard to bacterial pathogens, Scortichini et al. (27) described the association of several bacterial species with the decline of *Q. cerris* in central Italy, with *P. agglomerans* (*Erwinia herbicola*) being the species most frequently isolated from necrotic and healthy tissues. The role of *Pantoea agglomerans* in the development of the observed symptoms is still unknown because the isolated bacteria were not tested for pathogenicity. Barnard et al. (3) described the widespread occurrence of *Xylella fastidiosa* in Florida on populations of *Q. laevis* exhibiting decline or leaf scorch symptoms and the infrequent detection on asymptomatic trees. These authors used only a serological technique for detection and did not test the pathogenicity of *X. fastidiosa* isolates on oak; therefore, the causality of this bacterium in the observed symptoms remains to be confirmed.

Erwinia quercina, first described by Hildebrand and Schroth (12) as the etiological agent of drippy nut disease in *Q. agrifolia* and *Q. wislizenni*, recently was reclassified as *Brenneria quercina* by Hauben et al. (11). This bacterium was isolated from oozing and sticky acorns. The ooze was observed at the base of the nut and later on the acorn cup, after the nut had fallen (12). The disorders described by these authors were restricted to the nut and, although they recovered the bacterium from the surface of oak leaves, no symptoms on any other part of oak trees were reported. Little is known about this bacterial pathogen or the disease that it causes (20,35). To our knowledge, the occurrence of drippy nut of

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oak has not been reported since its first description in 1967 by Hildebrand and Schroth (12).

During the past decade, oak species with decay symptoms and cankers have been found in several forests in central Spain. In all cases, bark cankers with exudates were observed. We describe here the first isolation of *B. quercina* from bark cankers on two *Quercus* species and the characterization of Spanish isolates compared to reference strains of other *Brenneria* spp. Furthermore, we describe new symptoms not yet associated with this bacterial pathogen.

MATERIALS AND METHODS

Symptoms and bacterial isolation. The affected trees were found in four forests located in central Spain. Necrotic outer and inner bark tissue samples from longitudinal cankers on the trunk bark and oozing acorns and buds were analyzed during spring and/or summer 1992, 1995, and 1996 (Table 1). Small sections in the border of necrotic and healthy tissues were aseptically excised, comminuted, and submerged in 10 mM phosphate-buffered saline (PBS), pH 7.2, and directly streaked (or placed after dilutions) on King's B medium (14) plus cycloheximide (250 mg/ml). After incubation for 48 to 72 h at 25°C, *Brenneria*-like colonies were selected and purified. Thirty-five isolates were obtained from trunk cankers, acorn, or bud exudations from *Q. ilex* and *Q. pyrenaica* trees.

Physiological and biochemical characterization. Bacterial strains were grown routinely in King's B medium (14) and incubated at 25°C. They were presumptively identified to the species level by their Gram reaction, Kovac's oxidase, oxidative or fermentative metabolism, aesculin hydrolysis, urease and aminopeptidase activities, indol and levan production, pectate degradation, sucrose-reducing compounds, and hypersensitive reaction in tobacco leaves, as described by Schroth and Hildebrand (25). Two isolates from each origin were selected to confirm identification at the species level by using the multitube API 20E and API 20NE systems as recommended by the manufacturer (BioMérieux, Marcy-l'Etoile, France). The API 50CH system also was used but the inorganic medium of Ayers et al. (2) with 0.015% bromothymol blue was used as the basal medium to optimize the reactions. All the inoculated API strips were incubated at 25°C and read after 24 and 48 h. One reference strain of *B. quercina* from *Quercus* sp. CFBP 1266, and reference strains of other *Brenneria* species (11) were used as controls (Table 1). All isolates were assayed at least twice.

Fatty acid profiles. Qualitative and quantitative cellular fatty acid analyses were conducted as described previously by Sasser (24). Fatty acid methyl esters (FAMEs) were extracted using hexane/methyl-tert butyl ether (MTBE) and analyzed with the Microbial Identification System (MIS, Hewlett-Packard model 5898) and the corresponding software (MIDI; Microbial ID, Inc. Newark, DE). Fatty acid profiles of selected isolates were compared with the reference strain CFBP 1266. Two chromatographic runs were made with the extract of each sample and the extractions were repeated at least twice. Individual FAMEs were quantified and identified using the peak-naming table component of the MIS software package. Quantities were expressed as percentages of the total named FAME peak area. The mean and standard deviation for each FAME were determined. FAMEs are referred to below as the parent fatty acids.

Serological characterization. The Spanish isolates and the reference strain of *B. quercina* CFBP 1266 were analyzed by indirect enzyme-linked immunosorbent assay (ELISA-I) and indirect immunofluorescence (IF-I), as described by Hampton et al. (10). Rabbit antisera were raised against whole cells and O-antigen of the reference strain of *B. quercina* CFBP 1266 and against whole cells of the Spanish isolate 1467a. The schedule of immunization was as follows: five intramuscular injection of 1 ml (10^9 CFU/ml) weekly, except for one intravenous injection on day 21. Antisera were obtained 45 days after the first injection. Antisera titer was determined by ELISA-I and IF-I as described before (10). IF-I and ELISA-I were carried out in duplicate for each isolate and antiserum. PBS and *Pseudomonas syringae* pv. *syringae* 773-1 (from the Instituto Valenciano de Investigaciones Agrarias collection) were used as negative controls for IF-I and ELISA-I, respectively.

Pathogenicity tests. One isolate from each origin was selected for pathogenicity assays on acorn, using five acorns per bacterial isolate. The reference strain of *B. quercina* CFBP 1266 and PBS were used as positive and negative controls, respectively. Mature acorns were rinsed, disinfected for 5 min with a solution of 0.5% sodium hypochloride and 3% ethanol (wt/vol), and rinsed again with sterile distilled water prior to inoculation, as previously described by Hildebrand and Schroth (12). Several attempts were made to reproduce the symptoms caused by *B. quercina*. First, acorns detached from holm oak were punctured with a needle, a drop of a bacterial suspension in PBS (10^9 CFU/ml) was placed in the wound, and the inoculated nut was incubated in a sterile moist chamber at 29°C, as described by Hildebrand and Schroth (12). In a second method, disinfected acorns attached to small branches were immersed in a bacterial suspension (10^9 CFU/ml), placed in sterile 1% agar (wt/vol) in closed 500-ml bottles, and incubated at 25°C. Symptoms appearance was monitored for 2 weeks. Bacteria were reisolated on King's B medium (14). The experiments were repeated at least once.

Trunk inoculations were conducted on *Q. ilex* and *Q. pyrenaica* trees grown in pots or identified in the forest. The group of potted trees consisted of 288, 3- to 5-year-old, *Q. ilex* and *Q. pyrenaica* specimens. The 72 oak trees tested in the forest were at least 20 years old. *Q. ilex* and *Q. pyrenaica* trees were inoculated in the summer with one representative bacterial isolate from each host species, 1442-2b from holm oak and 1467a from *Q. pyrenaica*. Groups of 24 young plants or 6 mature trees were inoculated per strain and the experiments were performed in duplicate. The

TABLE 1. Origin of analyzed *Quercus* samples and reference strains of *Brenneria* spp. used

Bacterial sample, strain[a]	Host	Origin	Isolation date	Tissue source	Symptoms
1251 (6 isolates)	*Q. ilex*	El Pardo (Madrid)	1992	Trunk bark	Exudative canker
1442 (8 isolates)	*Q. ilex*	El Pardo (Madrid)	1995	Trunk bark	Exudative canker
1467 (6 isolates)	*Q. pyrenaica*	El Escorial (Madrid)	1995	Trunk bark	Exudative canker
1618 (5 isolates)	*Q. ilex*	Riofrío (Segovia)	1996	Acorns	Drippy nuts
1625 (10 isolates)	*Q. pyrenaica*	Soto del Real (Madrid)	1996	Buds	Drippy buds
B. quercina CFBP 1266	*Quercus* sp.	California, USA	1967	Acorns	…
B. alni CFBP 3923^T	*Alnus cordata*	Italy	1996	Trunk bark	…
B. nigrifluens NCPPB 564^T	*Juglans regia*	California, USA	1957	Trunk bark	…
B. paradisiaca CFBP 3477^T	*Musa paradisiaca*	Colombia	1970	Roots	…
B.rubrifaciens NCPPB 2020^T	*Juglans regia*	California, USA	1966	Trunk inner bark	…
B. salicis CFBP 802^T	*Salix alba caerulae*	United Kingdom	1939	Xylem	…

[a] CFBP = Collection Française de Bactéries Phytopathogènes, INRA, France; T = type strain; NCPPB = National Collection of Plant Pathogenic Bacteria, York, England.

Californian strain CFBP 1266 and PBS were used as positive and negative controls, respectively. The bark surface was disinfected with 95% ethanol prior to making a transverse cut with a disinfected scalpel into the bark. The bacterial suspension in PBS (10^9 CFU/ml) was injected into the wound (25 to 50 µl and 400 µl for young and old trees, respectively), and the wounds were sealed with plastic film. Plants in pots were maintained in the open air. The appearance of external and internal symptoms was recorded before and after removing the bark and measuring the length of the affected tissue 3 and 28 months after inoculation. Bacteria were recovered from symptomatic plants on King's B medium (14).

Statistical analysis. Statistical analysis of pathogenicity experiments data was performed using the statistical analysis package SPSS/PC (version 9.0 for Windows; SPSS Inc., Chicago). The Fisher's Exact Test for categorical data was used to study possible differences in symptoms occurrence between the following groups: (i) trees inoculated with strains from *Q. ilex* or *Q. pyrenaica*, (ii) young or mature oak trees, (iii) *Q. ilex* or *Q. pyrenaica* trees, and (iv) trees with symptoms observed 3 or 28 months after inoculation in each of above-mentioned groups. Statistical significance was accepted for all values of P lower than 0.05.

RESULTS

Symptomatology. Unlike the drippy nut disease described in California, the first symptoms observed in Spain were bark cankers, but the drippy nut was observed in subsequent surveys. A frequent symptom was the presence of irregular longitudinal cankers of a few centimeters in size on the trunk (Fig. 1) and branches (Fig. 2), reaching in some cases to 20 cm. These cankers, variable in depth, and showing necrosis of the affected tissues and copious exudation, usually appeared on the bark surface of the lower trunk. Necrotic lesions extended to inner bark tissues, reaching the pith in the smaller branches. In some cases, from small cankers of only few centimeters, we observed extensive internal lesions. The exudates, observed mainly during autumn and spring, were variously colored from white and frothy to a reddish or brownish color. They gradually became darker as they dried, and the surrounding bark turned reddish and finally dark brown or blackish. Oak trees seriously affected by bark cankers were usually mature (more than 20 years old), showing a progressive loss of vigor, foliage reduction, and early leaf senescence.

Exudates frequently were observed in growing acorns. This copious, sticky, honey-like sap appeared under the acorn cup (Fig. 3) and was responsible for severe fruit drop. Consequently, infected trees produced fewer acorns, most of which rotted and fell from the acorn cup. Exudations from leaf buds also were observed in some affected *Q. pyrenaica* trees, a symptom not previously described as being caused by *B. quercina*.

Bacteriological analysis. Bacterial colonies with morphological characters similar to *B. quercina* were isolated on King's B medium from trunk cankers and from acorn and bud exudations from *Q. ilex* and *Q. pyrenaica*. Almost pure cultures frequently were obtained. Colonies were cream in color, slightly convex, and circular, with entire margins and a shiny transparent texture, and did not produce pigments on this medium. Isolates initially were identified as belonging to the species *B. quercina* according to Hildebrand and Schroth (12) and Hauben et al. (11), because they were gram negative, oxidase negative, facultatively anaerobic, aesculin positive, urease negative, and produced sucrose-reducing

Fig. 1. Trunk cankers with dark exudates staining the bark of a *Quercus ilex* tree seriously affected by *Brenneria quercina*.

Fig. 2. Bark canker on branch of a *Quercus ilex* tree exhibiting symptoms of *Brenneria quercina*, with necrotic lesions and dark exudates.

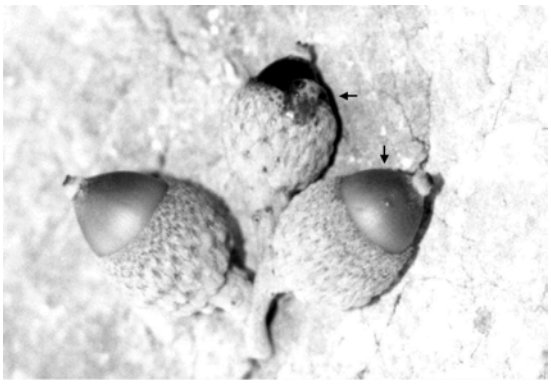

Fig. 3. Copious oozing of bacteria and sap from growing acorns of a *Quercus ilex* tree (the arrows indicate two acorns with ooze).

compounds, aminopeptidase, and mucoid growth in levan medium. They grew in King's B broth (14) at 37°C, were negative for nitrate reduction and pectate degradation, and did not induce a hypersensitive response in tobacco leaves.

Physiological and biochemical characterization. Ten selected isolates from five origins were analyzed further by API 20E, API 20NE, and API 50CH tests and compared with strain CFBP 1266 of *B. quercina* and the reference strains of other *Brenneria* species. The API 20E and API 20 NE systems revealed that all Spanish isolates had similar biochemical characteristics and resembled the reference strain CFBP 1266 (Table 2). In the API 20E strip, some false positive results were observed in acid production from inositol, rhamnose, melibiose, and arabinose after 48 h by all assayed isolates when compared with the results from the API 50CH and those from the literature (11–13). Thus, only the 24-h results for acid production were recorded (Table 2). The Spanish oak isolates differed in several tests of the API 20E and API 20NE strips from the reference strains of *B. alni*, *B. nigrifluens*, *B. rubrifaciens*, *B. paradisiaca*, and *B. salicis* (Table 2).

The modified API 50CH system also revealed homogeneous profiles for Spanish oak isolates, all of them being able to produce acid within 48 h from ribose, glucose, D-fructose, D-mannose, mannitol, *N*-acetylglucosamine, arbutine, esculine, salicin, sucrose, and gluconate, but not from erythritol, D-arabinose, L-arabinose, D-xylose, L-xylose, adonitol, L-sorbose, rhamnose, dulcitol, inositol, sorbitol, α-methyl-mannoside, cellobiose, maltose, lactose, melibiose, inulin, α-D-melezitose, D-raffinose, starch, glycogen, xylitol, β-gentibiose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, 2-keto-gluconate, and 5-keto-gluconate. The reference strain CFBP 1266 also acidified glycerol and galactose, sugars utilized by 5 and 8, respectively, out of 10 Spanish strains. These results also confirm the identification of Spanish oak isolates as *B. quercina*, according to Hildebrand and Schroth (12) and Hauben et al. (11).

TABLE 2. Physiological and biochemical characteristics of the Spanish oak isolates and reference strains of *Brenneria quercina* and other *Brenneria* species using the API 20E and API 20NE systems[a]

		Reference strains					
Characteristic	Oak[b] isolates	*B. quercina* CFBP 1266	*B. alni* CFBP 3923	*B. nigrifluens* NCPPB 564	*B. paradisiaca* CFBP 3477	*B. rubrifaciens* NCPPB 2020	*B. salicis* CFBP 802
API 20E							
ONPG test	–	–	–	–	+	–	–
Arginine dihydrolase	–	–	–	–	–	–	–
Lysine decarboxylase	–	–	–	–	–	–	–
Ornithine decarboxilase	–	–	–	–	–	–	–
Simmons citrate	+	+	–	–	+	–	–
Production of							
H_2S	–	–	–	–	–	–	–
Urease	–	–	–	–	–	–	–
Tryptophane desaminase	–	–	–	–	–	–	–
Indole	–	–	–	–	+	–	–
Voges-Proskauer	+	+	+	–	+	–	+
Gelatin liquefaction	–	–	–	–	–	–	–
Acid production from[c]							
Glucose	+	+	+	+	–	+	+
Mannitol	+	+	+	+	–	+	+
Inositol	–	–	–	+	–	–	+
Sorbitol	–	–	–	+	–	–	–
Rhamnose	–	–	–	+	+	–	–
Sucrose	+	+	+	+	+	+	+
Melibiose	–	–	–	+	+	–	–
Amygdalin	+	+	+	+	+	–	–
Arabinose	–	–	+	+	+	+	–
API 20 NE							
NO_3^- reduction	–	–	–	–	+	–	–
NO_2^- reduction	–	–	–	–	–	–	–
Indole production	–	–	–	–	+	–	–
Acid from glucose	+	+	+	+	+	+	+
Arginine dihydrolase	–	–	–	–	–	–	–
Urease	–	–	–	–	–	–	–
Aesculin hydrolysis	+	+	+	+	+	–	+
Gelatin liquefaction	–	–	–	–	–	–	–
PNPG test	v	–	–	–	+	–	–
Utilization of							
Glucose	+	+	+	+	+	+	+
Arabinose	–	–	+	+	+	+	–
Mannose	+	+	+	+	+	+	–
Mannitol	+	+	+	+	+	+	+
N-Acetyl-glucosamine	+	+	+	+	+	+	+
Maltose	–	–	+	–	–	–	–
Gluconate	+	+	+	+	+	+	+
Caprate	–	–	–	–	–	–	–
Adipate	–	–	–	–	–	–	–
Malate	+	+	+	+	+	+	+
Citrate	+	+	–	–	+	–	–
Phenyl-acetate	–	–	–	–	–	–	–

[a] CFBP = Collection Française de Bactéries Phytopathogènes, INRA, France and NCPPB = National Collection of Plant Pathogenic Bacteria, York, England. Strain possesses character (+), does not possess character (–), or variable (v).
[b] Ten Spanish oak isolates.
[c] After 24 h of incubation at 25°C.

Fatty acid profiles. The mean percentages of peak areas for all the major FAMEs are shown in Table 3. They include the saturated fatty acids 14:0 and 16:0, the monosaturated fatty acids 16:1 w7c and 18:1 w7c, the cyclopropane fatty acid 17:0 cyclo, and the hydroxy fatty acids 14:0 3OH. The minor fatty acids were 12:0, 15:0, 18:0, 19:0 cyclo, 15:0 iso 3OH, and an as-yet-unknown fatty acid with an equivalent chain length of 14.503. Minor FAMEs that were present at low values or only in one or two strains were omitted. The total peak area of the gas chromatography profiles was within the range of values that avoided the loss of peaks with less than 1% of the total named peak area. This was assessed in a previous assay using lipids from different weights of fresh cells. The reproducibility of the profiles was good and standard deviations were low (Table 3).

The Spanish strains and the reference strain were not identified as *Erwinia* (*Brenneria*) *quercina* as the first choice of the MIS; they were identified as *Edwarsiella hoshinae*, *Proteus vulgaris,* or *Pasteurella haemolytica*. However, six of these strains were identified as *Erwinia quercina* as second or third or fourth choice with similarity indices between 0.58 and 0.71.

Serological analysis. The working dilutions of the three antisera in IF-I and ELISA-I were 1:1,000 and 1:25,000, respectively, for AS-1266-WC; 1:400 and 1:5,000, respectively, for AS-1266-O (prepared with the Californian strain); and 1:300 and 1:25,000, respectively, for AS-1467a-WC (prepared with the Spanish strain). The reactions of the Spanish oak isolates and the reference strain 1266 in IF-I and ELISA-I with the three antisera are shown in Table 4. Antisera obtained with strain CFBP 1266 (using whole cells and somatic antigen) and isolate 1467a (using whole cells) showed a high reactivity with their homologous strains used for rabbit immunization as well as with the heterologous ones. AS 1266-WC, AS 1266-O, and AS 1467a-WC reacted with 8, 8, and 7 out of 10 Spanish isolates of *B. quercina*, respectively, by IF-I and with 5, 8, and 10 out of 10 isolates, respectively, by ELISA-I. None of three antisera reacted with all isolates by IF-I at the dilutions tested (Table 4). By ELISA-I, only the antiserum for the Spanish isolate 1467a reacted with all Spanish isolates of *B. quercina.* Some differences were observed by IF-I between antisera obtained from whole cells and from somatic antigen of strain CFBP 1266, suggesting differences in surface antigens. Results obtained with the three antisera and the two serological techniques showed serological heterogeneity among Spanish oak isolates from different origins.

Pathogenicity tests. Oozing was not observed 2 weeks after inoculation of needle wounds on holm oak nuts. However, ooze was observed within a week on acorns inoculated by immersion in suspensions of the Spanish isolates and the Californian strain of *B. quercina.* Bacterial isolates having the same characteristics as those inoculated were recovered from nut oozing.

The results of inoculating pot-grown or field-grown oaks with the bacterial isolates are given in Table 5. Three months after inoculation, external cankers like those observed under natural

TABLE 3. Fatty acid composition (percentage of peak areas) of *Brenneria quercina* Spanish isolates from *Quercus ilex* and *Q. pyrenaica*[a]

Fatty acid class	CFBP 1266	1251 (*n* = 2)	1442 (*n* = 2)	1467 (*n* = 2)	1618 (*n* = 2)	1625 (*n* = 2)
Saturated fatty acids						
12:0	0.57 (0.0)	0.59 (0.04)	0.51 (0.03)	0.57 (0.02)	0.49 (0.03)	0.56 (0.07)
14:0	11.20 (0.13)	11.19 (0.33)	10.22 (0.15)	10.18 (0.20)	10.25 (0.18)	10.07 (0.05)
15:0	0.46 (0.07)	0.13 (0.05)	0.86(0.40)	0.50 (0.20)	0.80 (0.09)	0.72 (0.20)
16:0	33.92 (0.37)	33.42 (0.61)	33.31 (0.36)	32.50 (1.02)	33.39 (0.62)	32.78 (0.14)
18:0	0.30 (0.03)	0.34 (0.40)	0.49 (0.11)	0.39 (0.07)	0.38 (0.04)	0.35 (0.02)
Unsaturated fatty acids						
16:1 w7c	23.93 (1.83)	25.48 (1.08)	26.48 (1.04)	26.86 (1.08)	27.68 (1.18)	27.97 (1.13)
18:1 w7c	8.15 (0.91)	11.77 (1.12)	11.70 (0.74)	12.04 (0.82)	11.91 (0.90)	12.16 (0.66)
Cyclopropane fatty acids						
17:0	8.92 (1.17)	6.34 (1.28)	4.58 (0.79)	4.52 (0.67)	4.39 (0.52)	3.81 (0.47)
19:0	1.40 (0.31)	1.17 (0.24)	0.73 (0.16)	0.69 (0.20)	0.77 (0.17)	0.63 (0.04)
Hydroxy fatty acids						
14:0 3OH	9.41(0.2)	8.20 (0.24)	9.03 (0.08)	9.05 (0.27)	9.28 (0.16)	9.29 (0.19)
15:0 iso 3OH	0.42 (0.13)	0.82 (0.18)	0.45 (0.19)	0.58 (0.08)	0.06 (0.06)	0.11 (0.06)
Unknown 14.503[b]	0.80 (0.0)	0.95 (0.04)	0.81 (0.06)	0.80 (0.03)	0.88 (0.1)	0.35 (0.18)

[a] CFBP = Collection Française de Bactéries Phytopathogènes, INRA, France; *n* = number of isolates assayed per each sample. Mean (%) and standard deviation of two extractions of two strains per each sample.
[b] Equivalent chain length relative to the lengths of known fatty acids.

TABLE 4. Indirect immunofluorescence (IF-I) and indirect enzyme-linked immunosorbent assay (ELISA-I) reactivities of *Brenneria quercina* strains with antisera against strains CFBP 1266 from California and 1467a from Spain[a]

	IF-I reactivity with antiserum[b]			ELISA-I reactivity with antiserum[c]		
	AS 1266		AS 1467-a	AS 1266		AS 1467-a
Strain	WC	O	WC	WC	O	WC
CFBP 1266	+++	+++	++	+++	+++	+++
1251-3	++	+	+	++	++	+++
1251-5	++	+	++	+++	+++	+++
1442-2	+	+	+	–	–	++
1442-8	+	–	+	+	+++	+++
1467-a	++	–	++	+++	+++	+++
1467-h	++	+	+	++	+++	+++
1618-a	+	+	–	–	+	++
1618-e	–	+	+	–	–	+
1625-1	+	+	–	–	++	++
1625-2	–	+	–	–	+	+

[a] CFBP = Collection Française de Bactéries Phytopathogènes, INRA, France; WC = antiserum prepared against whole bacterial cells; O = antiserum prepared against O antigen.
[b] +++ = Similar reaction to the homologous strain; ++ = weaker reaction than the homologous; + = weak reaction; – = negative reaction.
[c] +++ = Optical density at 405 nm (OD_{405nm}) ≥ 2 ; ++ = 2 < OD_{405nm} ≤ 1; + = 1 < OD_{405nm} > 0.5; – = OD_{405nm} ≤ 0.5, negative control = OD_{405nm} ≤ 0.25.

conditions were not visible, but small areas of necrotic tissue appeared in the bark above and below the inoculation points. When the bark was removed, brown necrotic streaks from 5 to 35 mm in length in young plants and from 20 to 150 mm in length in mature trees were observed around the inoculation sites in *Q. ilex* and *Q. pyrenaica*. Bacteria that resembled those used in inoculations were recovered from the lesions (Table 5). Thus, the Spanish isolates and the reference strain CFBP 1266 from drippy nut were able to produce internal symptoms in holm oak and *Q. pyrenaica*, irrespective of their origin. No differences were observed between isolates coming from *Q. ilex*, *Q. pyrenaica*, or California in their ability to produce lesions in any of the species assayed. Typical external cankers were not observed in most of the oak trees after 3 months; therefore, half of the inoculated trees were analyzed after 28 months. In general, trees did not show a progression of external symptoms, except for a few potted trees in which cracking of the trunk bark and development of external cankers with exudates occurred. *B. quercina* was reisolated from these cankers. No differences were found in the length of the inner lesions on oak trees growing in the field analyzed after 3 or 28 months, which ranged from 25 to 150 mm for *Q. ilex* and from 20 to 120 mm for *Q. pyrenaica*, irrespective of the origin of the inoculated strain. However, the length of the inner affected tissues on young pot-grown oaks after 28 months progressed up to 145 mm. Statistical analysis of data showed significant differences ($P < 0.05$) in symptom occurrence in the 3- to 5-year-old holm oak trees analyzed 3 or 28 months after inoculation, regardless the strain assayed (Table 5). Thus, for pathogenicity experiments using young holm oaks, trees should be analyzed after more than 3 months and, when possible, after 2 years. The majority of inoculated trees showed similar internal lesions and reisolated strains were identified as *B. quercina* (Table 5).

DISCUSSION

The bacterium causing exudative bark cankers in Spanish oak forests originally was isolated in 1992 from holm oaks (*Q. ilex*). The symptoms consisted of bark cankers with dark and sticky exudates, which had been observed over several years. Similar lesions on the trunk of walnut trees and alders are caused by species belonging to the genus *Brenneria* (*Erwinia*), such as *B. nigrifluens* (11,33), *B. rubrifaciens* (11,34), and *B. alni*, respectively (11, 29). They were described as responsible for bark canker and phloem canker of Persian walnut trees and bark canker of alder trees. The main symptom of such diseases is a dark, exudative canker on the trunk bark, which is limited to the outer bark for *B. nigrifluens* (33), to the inner bark and cambium for *B. rubrifaciens* (34), and to the bark for *B. alni* (29). *B. nigrifluens* and *B. rubrifaciens* recently have been identified, for the first time in Europe, as causing trunk cankers on Spanish trees (9,17). In diseased oak trees, the symptoms looked very similar, with longitudinal cracks extending through the affected areas and with ooze sometimes escaping from the canker and dripping over the bark surface. We have maintained the name canker for the exudative lesions observed on oaks trees, although they are not typical bacterial cankers. From these oaks, we isolated a bacterium with morphological and physiological characteristics similar to those reported for *B. quercina* (11–13), which differs from *B. nigrifluens*, *B. rubrifaciens*, and *B. alni* in several tests. To our knowledge, *B. quercina* had never been identified outside California nor associated with bark cankers.

Samples of exudative cankers from different origins were analyzed and similar bacteria were isolated in 1995 from *Q. ilex* and *Q. pyrenaica*, demonstrating a consistent association of *B. quercina* with cankers. Some attempts were made to isolate fungal pathogens from these samples, looking for those previously associated with the oak decline in Spain and other countries (18–21). No fungi were associated with bark cankers, drippy nut, or drippy bud and no *Phytophthora* sp. was isolated from the roots or the surrounding soil of the affected trees (28).

B. quercina was described by Hildebrand and Schroth as the causal agent of the drippy nut disease of California live oaks (12). The sole symptom described by these authors was a copious oozing of plant sap from the acorns and some rotting of the nut. Surveys from 1996 in forests close to Madrid demonstrated that both symptoms were very widespread in all the surveyed areas. An initial damage evaluation revealed that approximately 30 to 40% of the acorns were affected (28). Acorns are a valuable food source for wild life and are harvested for feeding the Iberian pig; therefore, the economic losses caused by this disease are relevant.

When comparing the Spanish isolates from bark cankers, as well as acorn and bud exudations, with the Californian strain CFBF 1266 by means of the API 20E and API 20 NE systems, we observed similar results in the utilization of sugars and other organic compounds. However, the Spanish isolates were clearly different from the other reference strains of *Brenneria* spp. assayed (Table 2). With API 50CH strips, we also observed homogeneous profiles for the Spanish isolates and the reference strain of *B. quercina.* However, differences in acid production from some sugars were observed between the API 20E (after 48 h) and the API 50CH results. They could be due to the different amount of bromothymol blue used in the API 20E gallery or to the modified basal medium. To increase the repeatability of the results with the API 50CH, we changed the commercial basal medium to Ayers medium and increased the amount of bromothymol blue to 0.015% (wt/vol).

TABLE 5. Pathogenicity of *Brenneria quercina* strains for *Quercus ilex* and *Q. pyrenaica*

	Trees with symptoms/analyzed trees[a]											
	3 years old						>20 years old					
	Q. ilex			*Q. pyrenaica*			*Q. ilex*			*Q. pyrenaica*		
	Months			Months			Months			Months		
Strain	3	28	Reisol	3	28	Reisol	3	28	Reisol	3	28	Reisol
CFBP1266	3/9	4/4	ND	4/8	4/4	+	4/4	NT	+	2/3	NT	+
CFBP1266	2/9	3/4	ND	6/8	4/4	+	3/4	NT	+	2/4	NT	+
1442-2b	6/13[b]	9/9[b]	+	10/13	6/11	+	2/3	3/3	+	2/3	3/3	+
1442-2b	6/14[b]	9/9[b]	+	8/14	9/10	+	2/3	2/2	+	3/3	3/3	+
1467-a	4/13[b]	9/9[b]	+	7/13	5/9	+	1/3	2/3	+	3/3	2/3	+
1467-a	7/14[b]	9/9[b]	+	6/14	7/10	+	3/3	3/3	+	3/3	2/3	+
PBS	0/7	0/3	–	0/8	0/4	–	0/2	NT	–	0/2	NT	–
PBS	0/9	0/3	–	0/7	0/4	–	0/2	NT	–	0/2	NT	–

[a] Reisol = reisolation; ND = not determined; NT = nontested; + = reisolation of bacterial strains similar to those inoculated, confirmed by biochemical tests; – = no reisolation of *Brenneria*-like colonies.

[b] $P < 0.05$ for comparison between 3- and 5-year-old holm oaks analyzed for internal symptom occurrence 3 or 28 months after inoculation.

The fatty acid composition of the isolates studied indicated that they were members of the family *Enterobacteriaceae* (7,29) but did not differentiate the Californian strain from the Spanish isolates. The difficulties in using the MIS system for accurate identification of *B. quercina* could be due to the library utilized (7). However, the FAME profiles of the Spanish isolates and the Californian strain were very similar to those previously reported for the type strain of *B. quercina* NCPPB 1852^T (29), reinforcing the identification of Spanish oak isolates as belonging to this species.

The serological analysis of Spanish isolates of *B. quercina* revealed that most of them reacted against the three antisera, showing that they have many antigens in common, regardless of their geographical origin and host. However, some antigenic heterogeneity was observed among them in IF-I and ELISA-I. In this sense, Spanish isolates coming from trunk bark cankers (1251, 1442, and 1467) were more closely related than those coming from sap oozing from acorns and buds (1618 and 1625). Surprisingly, the Californian strain CFBP 1266 from drippy nut showed a higher degree of relatedness with isolates from bark canker than with the Spanish isolates from acorn and leaf bud exudations. Similar results were obtained with antiserum 1467a, which confirmed some antigenic heterogeneity between Spanish isolates from different origins.

Spanish isolates of *B. quercina* were able to reproduce the symptoms caused by this bacterium under natural conditions in excised acorns, although oozing was not as abundant as in natural acorns attached to trees, agreeing with previous studies (12). However, in contrast to the results of Hildebrand and Schroth (12), the expression of symptoms was observed only when excised acorns were inoculated by immersion, not by a needle, suggesting that the bacterium might prefer entry through some natural openings. Maybe the anatomic and physiological differences between the nuts of the Californian and the Spanish oak species could explain the different results obtained.

Trunk inoculations of oak trees growing in pots or in the field further demonstrated that all tested isolates, as well as the reference strain CFBP 1266, were pathogenic for holm oak and *Q. pyrenaica*. Differences in the length of internal lesions were found in young trees (analyzed after 3 or 28 months), but not for mature trees. This observation suggests that the lesions produced by this bacterium in young plants can progress for months or years after inoculation; whereas, in older oaks, they occur within the first few months after inoculation, at least under the conditions of the two experiments performed. Significant differences in symptom development on young holm oak trees suggested that the pathogen spreads slowly in them compared with older trees. In fact, the bark cankers detected in the surveyed areas always were observed in mature trees. We observed external cankers with exudates in just a few potted trees and they were similar to those caused by *B. quercina* under natural conditions. However, small necrotic bark areas around the inoculation points in the trees inoculated with the drippy nut pathogen were of similar size to those reported in pathogenicity assays in walnut trees injected with *B. nigrifluens* (33). Although artificial inoculation of *B. quercina* did not always lead to the formation of cankers, internal necrosis, discoloration and degradation of host tissues, or reisolation from tissues distant from the inoculation site, demonstrated that this bacterium is able to colonize oak tissues, cause lesions, and survive for long periods. In addition, difficulties in reproducing external cankers of a certain size frequently have been reported by researchers inoculating woody plants with plant-pathogenic bacteria, including *B. nigrifluens* and *B. rubrifaciens* (9,15,17,33). This difficulty could be due to differences between natural infection conditions and artificial inoculations, the inoculation period, or the physiological state of the tissues at the inoculation time and the following weeks. Finally, whether the external lesions observed under natural conditions are produced by or just used by *Brenneria* spp. to enter into the plant remains to be determined.

This article reports the first European isolation of *B. quercina* and its association with bark canker of oak trees. The Spanish isolates of *B. quercina* were found in exudative bark cankers on *Q. ilex* and *Q. pyrenaica* and dark-brown sap exudate on bark is one of the symptoms observed in trees with oak decline; therefore, we believe this bacterium may be one of the biotic factors involved in the syndrome affecting *Q. ilex* and *Q. pyrenaica* in Spanish forests. Its role in the symptomatology on oaks observed in other countries should be determined.

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