For. Path. 30 (2000) 247–263

ISSN 1437 – 4781

Pathogenicity of fungi isolated from *Quercus suber* in Catalonia (NE Spain)

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Summary

Thirty-four fungal species isolated from cork oak (*Quercus suber*) in Catalonia (NE Spain) during 1992–95 were tested for pathogenicity either in stem, leaf or root inoculations. Eleven species were found to be pathogenic on stem: *Biscogniauxia mediterranea*, *Botryosphaeria stevensii*, *Diatrype* cf. *stigma*, *Endothia gyrosa*, *Fusarium solani*, *Graphium* sp., *Ophiostoma quercus*, *Phomopsis* sp., *Phytophthora cinnamomi*, *Sporendocladia bactrospora* and an unidentified Coelomycete. Three fungi showed pathogenic effects on leaves: *Dendrophoma myriadea*, *Lembosia quercina* and *Phomopsis quercella*. No clear pathogenic effects were detected in the root inoculation experiment. Trunk pathogens were differentiated into two groups according to the effects induced in the inoculated plants; *B. stevensii*, *Phomopsis* sp. and *P. cinnamomi* caused the death of the inoculated plants and induced the formation of large cankers and vascular necroses. The other pathogenic species also produced severe cankers and vascular lesions, but no significant mortality was detected. Water stress increased the lesions caused by *B. mediterranea* and *Phomopsis* sp., but limited those of *P. cinnamomi* and the rest of the inoculated fungi. However, water stress did not significantly affect the damage caused by *B. stevensii*, which was the most virulent of the species tested. Leaf pathogens only showed their effects if the leaf cuticle was previously damaged. *Lembosia quercina* caused small dark lesions whereas *D. myriadea* and *P. quercella* produced large necrotic areas in well-watered plants. The lesions caused by the last two fungi were reduced by water stress.

1 Introduction

In the last four decades a decline-type disease of cork oak (*Quercus suber* L.) has spread through several regions of Spain. Preliminary studies associated the mortality of trees with the following pathogenic fungi: *Biscogniauxia mediterranea* (de Not.) Kuntze (= *Hypoxylon mediterraneum* [de Not.] Ces. and de Not.) (Oliva and Molinas 1984; Torres Juan 1985), *Botryosphaeria stevensii* Shoem. (anamorph: *Diplodia mutila* Fr. apud Mont.) (Luque and Girbal 1989; Muñoz et al. 1992) and *Phytophthora* spp. (Molinas and Oliva 1984; Brasier et al. 1993). Moreover, additional research has focused on abiotic factors such as climatic conditions (Montoya 1995) and other local site characteristics (Jacobs et al. 1993).

In an exhaustive revision, Franceschini et al. (1993) compiled a list of about 300 fungal species occurring on cork oak. This work reported about 100 pathogenic species, although most of them were considered by the authors as secondary or weakness pathogens. Later, Marras et al. (1995) established 16 taxa as the most relevant pathogen species in Sardinia (Italy). Nevertheless, it is widely accepted that *Armillaria mellea* (Vahl: Fr.) Kumm., *B. mediterranea*, *B. stevensii* and *P. cinnamomi* are the four main pathogens of cork oak in the Mediterranean region (Franceschini et al. 1993; Santos 1995; Bakry and Abourouh 1996; Muñoz et al. 1996; Robin et al. 1998). In addition, other locally abundant pathogens

Received: 18. 1. 2000; accepted: 27. 4. 2000; editor: T. Kowalski

may also be considered; for example, *Endothia gyrosa* (Schwein.: Fr.) Fr. (SANTOS 1995) and several leaf fungi (MARRAS 1962a; MARRAS 1963).

The purpose of this research was to evaluate the pathogenicity of fungal species isolated from cork oak in Catalonia during 1992–95. In order to assess the pathogenicity of the fungi in relation to the hydric status of the host plant, inoculations on stem, leaf and root were performed on plants subjected to two water treatments: adequate irrigation and water stress.

2 Material and methods

2.1 Plant material and fungal strains

One-year-old cork oak seedlings obtained from field-collected acorns were used in all pathogenicity tests. Acorns were sown in 1000 ml containers filled with a peat–vermiculite mixture (peat: Floratorf; Floragard, Oldenburg, Germany; vermiculite: Termita 2; Asfaltex, Valldoreix, Barcelona, Spain) in a 1 : 1 ratio (v/v). The substrate was amended with Osmocote Plus® (Grace-Sierra Spain, Tarragona, Spain) at a dose of 2.5 g/l (final pH 5.0). The plants were maintained in a greenhouse and irrigated twice a week to field capacity until the setting up of the assays. Air temperature inside the greenhouse ranged from 15 to 42°C during the experiments, and averaged 7°C higher than the outside temperature. At the time of inoculations the mean plant size was 30 cm tall with a 5 mm stem diameter measured 10 cm above the substrate level. Table 1 shows the 38 fungal strains used in the pathogenicity tests, grouped by inoculation experiment: stem (32), leaf (four) and root (six). The strains used in the experiments were obtained from cork oak trees surveyed in the regions of Barcelona and Girona between 1992 and 1995 (LUQUE 1997). *Fusarium solani* (Mart.) Sacc. and *Verticillium tenerum* (Nees ex Pers.) Link, although isolated from trunk, were also tested in root inoculations. Similarly, the pathogenicity of *P. cinnamomi* was evaluated by means of root and stem inoculations. All strains were maintained in potato-dextrose agar (PDA) plugs in tubes with sterile distilled water at 4°C before use, except for *P. cinnamomi*, which was maintained in corn meal agar slants at 15°C (both culture media from Difco Laboratories, Detroit, MI, USA). Before the inoculations, the strains were recovered by plating a mycelial plug in PDA and incubating it at 25°C for 7 days.

2.2 Water stress

Plants used in stem and leaf experiments were distributed in two groups, one submitted to water stress and the other to sufficient water supply. Hydric stress was initiated about 50 days before the inoculations using two additional groups of 180 noninoculated plants (90 stressed + 90 irrigated). Leaf water potential (Ψ_l) of each group was monitored once a week (twice in summer months) at 0830 h (local time). Water potential was measured in two leaves of five randomly selected plants using a pressure pump (Soil Moisture Equipment, Santa Barbara, CA, USA), following the methods of SCHOLANDER et al. (1965).

The amount of water supplied to stressed plants, both inoculated and noninoculated, was determined specifically for each measurement time, and with the objective of maintaining Ψ_l below −1.4 MPa, a value which indicates water stress in evergreen *Quercus* (OLIVEIRA et al. 1992; TERRADAS and SAVÉ 1992). In order to prevent the death of stressed seedlings, plants were rehydrated when mean Ψ_l values were lower than −2.2 MPa. Nonstressed plants were irrigated twice a week to field capacity to ensure appropriate water availability.

2.3 Pathogenicity tests

2.3.1 Stem inoculations

Plants of both irrigation treatments were inoculated in groups of 10 with a total of 32 fungal strains (Table 1), in a completely randomized experimental design. A superficial wound (15 mm × 4 mm) was made on the bark of each plant at 10 cm above the ground level. A mycelial plug (4 mm in diameter) obtained from the margin of a fungal colony was placed in the wound, with the mycelium facing the stem, and the wound was wrapped with Parafilm® (American National Can, Greenwich, CT, USA). Ten additional control plants were treated similarly with sterile PDA plugs.

2.3.2 Leaf inoculations

A completely randomized experimental design was set up in which seedlings were inoculated in groups of 10 plants with the four strains listed in Table 1. Three apparently healthy leaves from the top of each plant were selected. The upper leaf surface was cleaned with sterile distilled water and the cuticle was damaged with a needle (approximately 9 mm^2) in two out of the three leaves. The third nonwounded leaf was used to determine whether infections could develop without previous damage. A mycelial plug (4 mm in diameter) from the margin of a fungal colony was then placed on each leaf, covered with several pieces of sterile filter paper soaked with sterile distilled water and finally wrapped with Parafilm. Paper pieces and Parafilm were removed 2 weeks later and each leaf was appropriately marked for future identification. Ten additional control plants were inoculated with sterile PDA plugs using the same procedure.

2.3.3 Root inoculations

Root inoculations were performed only on plants with no water restrictions. Two weeks before the inoculations, plants were transplanted to a steam-sterilized peat–vermiculite substrate (1 h at 120°C). Inoculations were made in groups of 10 plants with the six selected strains in a completely randomized design (see Table 1). The inocula consisted of mycelial homogenates (DHINGRA and SINCLAIR 1985); two fungal colonies of each strain previously grown on solid media were blended in 120 ml of sterile distilled water for 10 s. Viability of the fungi was assessed by plating 1 ml of the homogenates in PDA and checking for further growth. Roots were damaged with a sterile blade, and 10 ml of the homogenates were applied directly to the roots. An additional control group of 10 plants was established by inoculating them with sterile media homogenates. Plants were maintained with subsequent periods of flooding (two per week) throughout the experimental period.

2.4. Monitoring and data analysis

Each pathogenicity test was run for 6 months, between August 1994 and February 1996. Symptom observations were made fortnightly and included foliar necroses, wilting or yellowing, stem canker, epicormic branching, and production of fungal fruiting bodies. Three months after the inoculations, half of the plants of each treatment were removed from the assays. Symptoms were annotated once again and plants were processed in the following ways:

Stem inoculations. The lengths of vascular necroses were measured upwards and downwards from the point of inoculation, and total necrosis was calculated. Fungal re-isolations were accomplished by plating in PDA several surface-sterilized wood pieces (3 min in 70° ethanol) taken from the necrotic tissues at intervals of 1 cm. Cultures were incubated at 25°C for further identification of the isolated fungi.

Table 1. **Fungal strains used in the pathogenicity tests on *Quercus suber***

Inoc. test	Taxa	Strain no.	Isolation source [1]	Isolation date	UTM location Zone 31 N (1 × 1 km) (*x y*)	
Stem	*Acrodontium crateriforme* (van Beyma) de Hoog	141	T	May 1994	489	4626
	Armillaria sp.	169	T	Jun 1995	492	4639
	Biscogniauxia mediterranea (de Not.) Kuntze	5	T	Oct 1992	506	4640
	Botryosphaeria stevensii Shoem.	58	T	Jan 1992	460	4608
	Brachydesmiella biseptata Arnaud ex Hughes	178	T	Jul 1995	486	4696
	Coryneum quercinum Muthumary and Sutton	145	B	Jun 1994	495	4648
	Coryneum umbonatum Nees ex Steudel	148	B	Jun 1994	471	4613
	Dactylaria purpurella (Sacc.) Sacc.	162	T	Jun 1995	484	4617
	Dendryphion comosum Wallr.	146	T	Jun 1994	495	4648
	Diatrype cf. *stigma* (Hoffm.: Fr.) Fr.	164	B	Jun 1995	492	4642
	Endothia gyrosa (Schwein.: Fr.) Fr.	88	T	Jul 1993	467	4637
	Fusarium solani (Mart.) Sacc.	91	T	Jul 1993	467	4635
	Fusarium solani (Mart.) Sacc.	138	T	May 1994	493	4648
	Fusarium solani (Mart.) Sacc.	143	T	Jun 1994	464	4631
	Graphium sp.	136	T	May 1994	471	4638
	Helminthosporium microsorum D. Sacc.	127	B	May 1994	485	4624
	Melanomma pulvis-pyrius (Pers.: Fr.) Fuckel	151	T	Jun 1994	471	4613
	Ophiostoma quercus (Georgew.) Nannf.	147	T	Jun 1994	464	4631
	Pestalotiopsis cf. *guepinii* (Desm.) Stey.	29	T	Apr 1993	506	4640
	Petriella guttulata Barron and Cain	161	T	May 1995	467	4623
	Phomopsis quercella (Sacc. and Roum) Died.	152	A	Jun 1994	462	4630
	Phomopsis sp.	167	B	May 1995	474	4620
	Phytophthora cinnamomi Rands	173	S	Jun 1995	492	4639
	Pyrenochaeta quercina Kabát and Bubák	93	T	Jul 1993	467	4635

Table 1. cont.

Inoc. test	Taxa	Strain no.	Isolation source [1]	Isolation date	UTM location Zone 31 N (1 × 1 km) (*x y*)	
	Pyrenochaeta quercina Kabát and Bubák	130	T	May 1994	488	4623
	Splanchnonema sp.	176	B	Jul 1995	457	4613
	Sporendocladia bactrospora (Kendrick) M. Wingfield	149	T	Jun 1994	464	4631
	Thyridaria macrostomoides (de Not.) Barr	180	T	Jul 1995	486	4696
	Verticillium tenerum (Nees ex Pers.) Link	128	T	May 1994	487	4625
	Verticillium sp.	179	T	Jul 1995	486	4696
	Unidentified Ascomycete	168	B	Jun 1995	492	4639
	Unidentified Coelomycete	183	B	Jul 1995	461	4615
Leaf	*Biscogniauxia mediterranea* (de Not.) Kuntze	76	L	May 1993	506	4640
	Dendrophoma myriadea [2] (Preuss) Sacc.	137	L	May 1994	487	4625
	Lembosia quercina (Ell. and G. Martin) Tracy and Earle	186	L	May 1994	490	4643
	Phomopsis quercella (Sacc. and Roum) Died.	152	A	Jun 1994	462	4630
Root	*Fusarium solani* (Mart.) Sacc.	91	T	Jul 1993	467	4635
	Fusarium oxysporum Schlecht.	185	R	Aug 1995	448	4596
	Phytophthora cinnamomi Rands	173	S	Jun 1995	492	4639
	Pythium sp. 1	39	R	Oct 1992	506	4640
	Pythium sp. 2	122	S	May 1994	491	4622
	Verticillium tenerum (Nees ex Pers.) Link	128	T	May 1994	487	4625

[1] All strains isolated from *Quercus suber*. Sources: A: acorn; B: branch; L: leaf; R: root; S: soil surrounding roots; T: trunk.

[2] According to Prof. B. C. Sutton (personal communication), the correct epithet for this fungus should be *Phomopsis glandicola* Grove. Nevertheless, we maintain the name of the first known occurrence on *Q. suber* (MARRAS 1962a), because of its further common acceptance (FRANCESCHINI et al. 1993; MARRAS et al. 1995) and the lack of a definitive identification for this species.

Leaf inoculations. Leaf necrotic area was estimated from the maximum and minimum necrosis diameter. Fungal re-isolations were carried out as indicated above, but with surface sterilization of leaf fragments limited to 20–30 s.

Root inoculations. Soil samples (approximately 250 ml) of the containers were baited with immature carnation petals to recover the inoculated fungi. The root system of each plant was carefully washed with tap water and the roots were later examined under a dissecting microscope. Root fragments showing abnormal symptoms (colour, turgor, etc.) were selected from each plant. Fragments were surface sterilized (0.5–2 min in 70° ethanol) and plated in CMA, acidified PDA and PARP (KANNWISCHER and MITCHELL 1978). Cultures were incubated at 25°C for further identification of the tested fungi.

At the end of the experiments, all the above actions were repeated. Data from the assays were analysed using SAS statistical package (SAS 1987). Data were checked for normality and equal variance distributions, transformed if necessary, and analysed using multifactorial analysis of variance (ANOVA) procedures. Data were grouped and analysed again if no interactions were found. After ANOVA, mean treatment values were compared against their respective controls with the Dunnett two-tailed test.

3 Results

3.1 Stem inoculations

Control plants from both irrigation treatments grew adequately and produced asymptomatic leaves during all trials, although growth of stressed seedlings was lower in comparison with the sufficiently irrigated plants. Regardless of water-supply level, wounds of all control plants were covered by calli within the experimental period, and no fungus was isolated from them. Table 2 summarizes the quantitative results obtained in the stem-inoculation experiment. Statistical analyses were made separately for each irrigation treatment and reading period due to significant interactions ($p \leq 0.05$) between all factors (data not shown). According to the results obtained, *Botryosphaeria stevensii*, *Phytophthora cinnamomi* and *Phomopsis* sp. were highly virulent. All three fungi caused the death of inoculated plants, large number of cankers, long vascular necroses, and were frequently re-isolated.

Botryosphaeria stevensii caused the death of nearly all plants in 7–14 days after the inoculations. Wilting of seedlings coincided with the production of dark, depressed cankers, and pycnidia on the bark surface. All plants were processed for symptom reading and pathogen re-isolation in order to avoid secondary fungal colonizations. Mean vascular necroses were the largest of all the inoculation treatments (21.0 and 18.0 cm for the normal irrigation and stress assay, respectively). The dimensions of these lesions were equivalent to 60% of mean plant height. Fungal colonization was, on average, about 2 cm larger than the necrosis. Water stress did not affect the lesions caused by *B. stevensii*, as shown by similar results obtained in both assays.

About half of the seedlings inoculated with *P. cinnamomi* died within 1 month after the inoculations regardless of the irrigation treatment. Cankers appeared in 19 plants and were characterized by a depressed and very dark-coloured bark. Vascular necrosis in dead plants monitored 3 months after inoculation was greater than that in surviving plants, which explains the differences observed in necrosis lengths between the two reading periods (see Table 2). Water stress significantly decreased mean necrosis length (about 80%), as well as the frequency of fungal recovery. Fungal colonization was strictly limited to the necrotic zones.

Phomopsis sp. caused the death of all the inoculated plants submitted to water stress. All inoculated plants regardless of the irrigation treatment developed bark cankers, although cankers were externally less apparent in water-stressed plants. Vascular lesions at

the end of the experiments were about 4 cm, slightly larger among stressed than non-stressed plants. Mycelium re-isolation was positive in nearly all inoculated plants of both irrigation treatments.

Only two plants inoculated with *Biscogniauxia mediterranea* and submitted to water stress died during the experimental period. Cankers were not externally apparent although vascular necroses were notable, especially in the water-stressed plants. Thus, the mean values at the end of the experiments were 2.8 cm for the normal irrigation assay and 5.4 cm for the water-stress experiment. Mycelial colonization was more extensive in stressed plants, and averaged 1.5 cm larger than the necrosis length.

Diatrype cf. *stigma* (Hoffm.: Fr.) Fr., *Endothia gyrosa* (Schwein.: Fr.) Fr., *Fusarium solani* (Mart.) Sacc., *Graphium* sp., *Ophiostoma quercus* (Georgew.) Nannf., *Sporendocladia bactrospora* (Kendrick) M. Wingfield and the unidentified Coelomycete (no. 183) caused numerous cankers, significant vascular necroses and were re-isolated very frequently. In general, they did not lead to the death of inoculated plants and their lesions were reduced under water-stress conditions (see Table 2 for exceptions). The three strains of *F. solani* used in the experiments behaved differently: strains 91 and 138 caused more cankers and longer necroses than strain 143, whereas water stress decreased significantly the vascular lesions of strains 138 and 143.

In comparison with the above fungi, the remaining inoculated strains produced relatively minor lesions. In general, plants did not die and showed small bark cankers (limited to 1–2 mm around the point of inoculation, with the exception of well-developed cankers due to *Coryneum quercinum* Muthumary and Sutton, *Pyrenochaeta quercina* Kabát and Bubák [no. 93] and *Phomopsis quercella* [Sacc. and Roum.] Died.). Mean vascular lesions were normally nonsignificant and shorter than 1 cm (except for *Pestalotiopsis* cf. *guepinii* [Desm.] Stey., *P. quercella* and the unidentified Ascomycete [no. 168]; see Table 2). Mycelial re-isolations were irregular, ranging from clearly positive (*Petriella guttulata* Barron and Cain and *Verticillium tenerum*) to absolutely negative (*Armillaria* sp., *Brachydesmiella biseptata* Arnaud ex Hughes and *Splanchnonema* sp.). There was also some variability in the response of these fungi to water stress: in general, water stress decreased development of canker and vascular necrosis as well as mycelial recovery. A clear increase in canker frequencies was noticed only in the plants inoculated with the unidentified Ascomycete (no. 168), but slight increases were also found in plants inoculated with *Armillaria* sp., *P. quercina*, *Thyridaria macrostomoides* (de Not.) Barr and *V. tenerum*.

3.2 Leaf inoculations

Foliar lesions caused by the inoculated fungi did not extend to other parts of the plant during the experiment. They usually consisted of circular, necrotic, brown-coloured areas. Table 3 summarizes the results obtained in this experiment. Analysis of variance showed a significant interaction of all factors ($p \leq 0.5$) except for the reading period (data not shown).

In the normal irrigation assay, *Dendrophoma myriadea* (Preuss) Sacc. was the only fungus that caused lesions in three out of 10 nonwounded leaves, although they were not statistically significant. In addition, mycelial recovery from nonwounded leaves was possible only for this fungus and *B. mediterranea*. On the other hand, only *D. myriadea* and *Phomopsis quercella* showed clear pathogenic effects on wounded leaves, as indicated by large necrotic areas (50 and 56 mm^2, respectively) and high percentages of mycelium recovery (95 and 70%, respectively). Necroses caused by *D. myriadea* were detected in 95% of the inoculated leaves and appeared 2 weeks after the inoculations. Those of *P. quercella* were noticed 4–8 weeks after the inoculations and developed on 80% of the leaves. The other fungi did not cause significant necroses although *B. mediterranea* was re-isolated from 50% of the wounded leaves.

Table 2 **Frequencies of symptoms, positive mycelial recovery and mean vascular necrosis of *Quercus suber* plants (n = 10) inoculated at the stem with several fungal species**

	Normal irrigation				Water stress			
Taxa[1]	Dead plants	Stem canker	Mycelial recovery	Necrosis[2] (cm)	Dead plants	Stem canker	Mycelial recovery	Necrosis (cm)
Control	0	0	0	0.1–0.2	0	0	0	0.1–0.1
Acrodontium crateriforme	0	0	5	0.3–**0.6**	0	0	0	0.2–0.2
Armillaria sp.	0	1	0	0.2–0.5	0	3	0	0.2–**0.7**
Biscogniauxia mediterranea	0	10	10	**2.7–2.8**	2	8	9	**5.7–5.4**
Botryosphaeria stevensii[3]	10	10	10	**21.0**	8	10	8	**18.2**
Brachydesmiella biseptata	0	3	0	0.3–0.1	0	2	0	0.3–0.1
Coryneum quercinum	0	5	6	**0.6–0.7**	0	6	3	0.4–**0.7**
Coryneum umbonatum	0	5	0	0.2–**0.6**	0	2	3	0.2–0.3
Dactylaria purpurella	0	2	4	0.2–0.3	0	2	2	0.3–0.1
Dendryphion comosum	0	1	2	0.3–**0.7**	0	1	1	0.1–0.1
Diatrype cf. *stigma*	0	10	7	**0.9–1.3**	0	6	1	**1.7–1.1**
Endothia gyrosa	0	10	10	**2.7–4.2**	1	7	10	0.9–**1.4**
Fusarium solani 91	0	10	10	**3.6–4.8**	1	10	10	**3.9–4.6**
Fusarium solani 138	0	7	10	**3.9–4.2**	0	8	10	**1.9–1.2**
Fusarium solani 143	0	5	10	**1.3–2.5**	0	2	7	0.3–0.4
Graphium sp.	0	5	10	**5.0–4.8**	0	9	9	1.1–**2.3**
Helminthosporium microsorum	0	2	1	**0.9–0.9**	0	2	0	0.3–0.3
Melanomma pulvis-pyrius	0	1	2	0.3–0.3	0	1	1	0.2–0.4
Ophiostoma quercus	0	9	9	**1.6–2.1**	0	5	10	0.6–**1.6**
Pestalotiopsis cf. *guepinii*	0	2	5	0.4–**1.2**	0	1	1	0.2–0.3
Petriella guttulata	0	4	10	**0.4**–0.5	0	3	7	0.2–**0.7**

Table 2. cont.

Taxa [1]	Normal irrigation				Water stress			
	Dead plants	Stem canker	Mycelial recovery	Necrosis [2] (cm)	Dead plants	Stem canker	Mycelial recovery	Necrosis (cm)
Phomopsis quercella	0	10	2	**1.3–2.5**	0	3	1	0.3–0.4
Phomopsis sp.	1	10	10	**1.8–3.7**	10	10	9	**3.2–4.0**
Phytophthora cinnamomi [4]	5	10	9	**9.4–3.4**	4	9	2	**1.6–0.8**
Pyrenochaeta quercina 93	0	3	8	**0.8–0.9**	0	4	0	0.3–**0.8**
Pyrenochaeta quercina 130	0	2	3	0.4–**1.0**	0	3	4	0.5–**1.0**
Splanchnonema sp.	0	5	0	0.1–0.3	0	1	0	0.1–0.1
Sporendocladia bactrospora	0	10	10	**2.7–4.1**	0	8	6	**1.5–1.1**
Thyridaria macrostomoides	0	4	2	0.3–0.4	0	6	0	0.2–0.2
Verticillium tenerum	0	2	7	0.4–0.3	0	4	8	0.8–**0.9**
Verticillium sp.	0	0	5	0.1–0.3	0	1	1	0.2–0.1
Unidentified Ascomycete 168	0	0	0	0.1–0.1	0	7	1	1.6–0.5
Unidentified Coelomycete 183	0	10	8	**0.9–1.0**	2	9	4	1.5–**1.6**

[1] With strain number when necessary.
[2] Necrosis length values at 3/6 months after inoculation. Significant differences from control by Dunnett test ($p \leq 0.05$) are shown in bold typeface. Original data were log-transformed for statistical analyses.
[3] Three weeks after inoculation. All plants processed.
[4] Only dead plants processed 3 months after inoculation.

Table 3. **Leaf necrosis area and mycelial recovery of *Quercus suber* plants (n = 10) inoculated with four fungal species**

	Normal irrigation				Water stress			
	Leaf wounded		Leaf not wounded		Leaf wounded		Leaf not wounded	
Taxa	Necrosis (mm^2) [1]	Mycelial rec. (%)	Necrosis (mm^2)	Mycelial rec. (%)	Necrosis (mm^2)	Mycelial rec. (%)	Necrosis (mm^2)	Mycelial rec. (%)
Control	11.3	0	0.0	0	19.9	0	0.0	0
Dendrophoma myriadea	**50.1**	95	2.0	30	15.8	100	0.3	10
Biscogniauxia mediterranea	4.6	50	0.0	10	21.0	85	0.0	0
Lembosia quercina	14.3	0	0.0	0	12.4	0	0.0	0
Phomopsis quercella	**56.0**	70	0.0	0	**32.5**	55	0.0	0

[1] Mean values corresponding to 20 wounded and 10 nonwounded inoculated leaves. Significant differences from control by Dunnett test ($p \leq 0.05$) are shown in bold typeface. Original data were log-transformed for statistical analyses.

In the water-stress assay, only *D. myriadea* was re-isolated from nonwounded leaves, but developed very small and nonsignificant necroses. On damaged leaves, *P. quercella* caused significant necroses and the percentage of mycelial recovery was over 50%. *Dendrophoma myriadea* and *B. mediterranea* were re-isolated in higher frequencies but no significant necroses were detected.

Lembosia quercina (Ell. and G. Martin) Tracy and Earle caused distinct lesions on the upper surface of leaves: small black spots, with a regular and defined margin (see MARRAS 1963). This fungus affected plants of both irrigation treatments, but only on wounded leaves. Mycelial recovery was not possible, probably due to an excessive sterilization of the leaf fragments during re-isolation.

3.3 Root inoculations

Mycelial homogenation did not affect fungal viability as shown by the positive colonial recovery of all homogenates (Table 4). Only one plant inoculated with *P. cinnamomi* died during the experimental period, and the rest of the inoculated plants behaved normally throughout the assay (i.e. grew and produced green and asymptomatic leaves). *Fusarium oxysporum* Schlecht., *F. solani*, *P. cinnamomi* and *Pythium* sp. were successfully recovered using the soil-baiting technique, but only the three former species were re-isolated from necrotic zones of the main and lateral roots; *P. cinnamomi* was re-isolated from eight plants, *F. solani* from nine, and *F. oxysporum* from all the inoculated plants. Necrotic areas of the main root, irrespective of the fungus inoculated, were sparse and measured only a few millimetres in size.

4 Discussion

Leaf water potential values of nonstressed plants, ranging from −0.7 to −0.3 MPa (mean: −0.4 MPa; SE 0.01 MPa), remained very stable during the entire experimental period. These values corresponded to well-watered plants and coincided with similar measurements made in natural conditions on nonstressed cork oak trees (OLIVEIRA et al. 1992). On the other hand, Ψ_l of stressed seedlings ranged from −3.0 to −0.4 MPa (mean: −1.3 MPa; SE 0.03 MPa), showing a fluctuating pattern. KSONTINI et al. (1998) observed a reduction of 50% in stomatal conductance of young *Quercus suber* plants when water potential was about −1.3 MPa, and a decrease of 70% at −1.7 MPa. On the other hand, TERRADAS and SAVÉ (1992) found that adult trees of *Quercus ilex* L., another Mediterranean evergreen

Table 4. **Number of plants showing infection symptoms and frequency of mycelial recovery of *Quercus suber* seedlings (n = 10) inoculated with six species of root fungi**

Taxa	Inoculum viability	Number of plants			
	With main root necrosis	Mycelium recovery from soil baiting	Mycelium recovery from roots		
Dead					
Control	−	0	0	0	0
Fusarium oxysporum	+	0	10	10	10
Fusarium solani	+	0	10	10	9
Phytophthora cinnamomi	+	1	10	10	8
Pythium sp. 1	+	0	2	2	0
Pythium sp. 2	+	0	3	10	0
Verticillium tenerum	+	0	0	0	0

oak, progressively closed their stomata when water potential values were lower than −1.3 MPa, and trees suffered from severe water stress below −2.2 MPa. As a conclusion, water restriction induced in *Q. suber* seedlings a moderate hydric stress punctuated by more drastic episodes, as indicated by the lowest readings registered during this study (−2.5 to −3.0 MPa). In relation to the development of the pathogenesis, water potential between −1.2 and −1.5 MPa predisposes the host to most fungal infections of weakness pathogens (SCHOENEWEISS 1978).

The results obtained in the stem-inoculation experiment show that *Botryosphaeria stevensii*, *Phomopsis* sp. and *Phytophthora cinnamomi* were the most virulent pathogens. They induced plant death and caused important canker and vascular lesions. *Botryosphaeria stevensii* was the most significant virulent pathogen: it wilted seedlings in 7–14 days after inoculations and showed the highest vascular colonization. LUQUE and GIRBAL (1989) reported the pathogenicity of *B. stevensii* to cork oak, but in nonstressed plants. Our present results indicate that *B. stevensii* maintains the same high level of virulence when the host is under water stress. LUISI et al. (1993) observed similar results, also irrespective of the water supply, in the inoculation of three *Quercus* species (*Q. cerris* L., *Q. pubescens* Willd. and *Q. trojana* Webb.) with *B. stevensii*. This fungus is known to be pathogenic to the following European species of *Quercus* in addition to cork oak: *Q. cerris*, *Q. ilex*, *Q. petraea* (Matt.) Liebl., *Q. pubescens*, *Q. robur* L. and *Q. trojana* (ROLAND 1945; VAJNA 1986; RAGAZZI and MESTURINO 1987; RAGAZZI et al. 1990; MUÑOZ et al. 1992; LUISI et al. 1993).

Phytophthora cinnamomi caused the death of about half of the inoculated plants regardless of the irrigation treatment, although the vascular necroses and the mycelial recovery were notably decreased when seedlings were subjected to water stress. ZENTMYER (1980) pointed out that the optimal water potential for the *in vitro* growth of this species occurs at the range of −1.0 to −1.5 MPa, whereas growth is reduced to a half when water potential is between −2.0 and −2.5 MPa. Maybe the lowest potentials registered during our experiment (approximately −3.0 MPa) negatively affected the growth and viability of this fungus, as shown by its low recovery frequency in the water-stress assay. *Phytophthora cinnamomi* is a well known pathogen of more than 900 plant species, including cork oak and other species of *Quercus* (MIRCETICH et al. 1977; ZENTMYER 1980). BRASIER et al. (1993), and recently GALLEGO et al. (1999), identified *P. cinnamomi* as an important contributory factor in the 'oak decline' of *Q. ilex* and *Q. suber* woodlands in SW Spain. ROBIN et al. (1998) also recognized the important role of *P. cinnamomi* in the decline after a survey for this pathogen in cork and holm oak forests in south-east France.

Phomopsis sp. was especially virulent in the water-stress assay, causing the death of all inoculated plants. However, canker production and vascular necroses were similar either in normally irrigated or in stressed plants. Several species of *Phomopsis* are considered as primary colonizers of twigs and are related with the natural pruning of trees (GRIFFITH and BODDY 1990; KOWALSKI and KEHR 1992; HALMSCHLAGER et al. 1993; KEHR and WULF 1993; SIEBER et al. 1995). Furthermore, some authors consider *Phomopsis* as moderately pathogenic, and associated with *Quercus* dieback and decline (KOWALSKI 1991; KEHR and WULF 1993; SIEBER et al. 1995). On the other hand, the results of the present work showed a moderate pathogenic behaviour of *P. quercella* in the trunk, thus coinciding with the opinion of MARRAS et al. (1995) and MUÑOZ et al. (1996).

Biscogniauxia mediterranea slightly increased the mortality of plants in the water stress assay, although vascular lesions (over 5 cm) were approximately twice that observed in the normal irrigation test. The pathogenic effects of this species on several Mediterranean species of *Quercus* are usually associated with stress episodes, such as summer drought (TORRES JUAN 1985; CAPRETTI AND MUGNAI 1987; RAGAZZI et al. 1989; VANNINI and SCARASCIA MUGNOZZA 1991). The latter authors observed an optimal growth of this fungus when xylem water potential of inoculated *Q. cerris* trees was at −2.0 MPa. VANNINI and VALENTINI (1994), working with the same host–pathogen combination, found a positive

relationship between stress conditions and the dimensions of the vascular necroses. Similar results were obtained by LUISI et al. (1993) with the inoculation of three *Quercus* species with *B. mediterranea*. However, later studies have shown that *B. mediterranea* can also induce negative effects even in the absence of water stress: loss of xylem hydraulic conductivity, reduced stomatal conductance and low trunk diameter growth (VANNINI and VALENTINI 1994; LUQUE et al. 1999).

Diatrype cf. *stigma*, *Endothia gyrosa*, *Fusarium solani*, *Graphium* sp., *Ophiostoma quercus*, *Sporendocladia bactrospora* and the unidentified Coelomycete (no. 183) were moderately virulent. They caused significant lesions but little seedling mortality. Water stress increased lesions of *D.* cf. *stigma* and the unidentified Coelomycete (no. 183), whereas it decreased those of *S. bactrospora*. To our knowledge, the specific pathogenicity of these fungi against *Q. suber* has not been proved yet, although some works consider the three former fungi as weak pathogens of cork oak (FRANCESCHINI et al. 1993; MUÑOZ et al. 1996).

The rest of the strains tested for pathogenicity on stem did not show remarkable effects. However, some of them are known by their pathogenicity against *Quercus*, such as *Armillaria* and *Ophiostoma*. *Armillaria* spp. (mainly *A. mellea* and *A. tabescens*) are frequently cited as cork oak pathogens (MARRAS 1962b; FRANCESCHINI et al. 1993; MARRAS et al. 1995; MUÑOZ et al. 1996). In our experiments, water stress slightly increased canker formation by *Armillaria* sp., although the fungus was never re-isolated. Similarly, ANSELMI and PUCCINELLI (1993) observed a significant increase in the lesions caused by *A. mellea* against six *Quercus* species when plants were water stressed. *Ophiostoma quercus* (also known as a sibling species within *O. piceae* [Münch] H. and P. Syd., see BRASIER 1993; BRASIER and KIRK 1993; BRASIER and STEPHENS 1993; HALMSCHLAGER et al. 1994; WULF and KOWALSKI 1994) occurs on several species of *Quercus*. It has been frequently associated with the 'oak decline' complex, although its pathogenicity has been often discussed (mainly as *O. piceae*, see DELATOUR 1983; OEPP/EPPO 1990; KOWALSKI 1991; BALDER 1993; DELATOUR et al. 1993). Our present results agree with those of BALDER (1993), who observed a reduction in vascular necroses due to *O. piceae* when inoculated seedlings of *Q. robur* were submitted to water stress.

Dendrophoma myriadea and *Phomopsis quercella* proved to be pathogenic to cork oak in the leaf inoculation experiment. Both fungi caused lesions four to five times greater than the control in the normal irrigation assay, but only if the leaf cuticle was damaged before the inoculations. Water stress affected both pathogens negatively, especially *D. myriadea*, which produced nonsignificant necroses. *Dendrophoma myriadea* is a known leaf pathogen of *Q. suber*, which was first noticed on cork oak trees in Sardinian forests (MARRAS 1962a). This fungus causes a characteristic marginal drying of leaves. However, MARRAS et al. (1995) considered *D. myriadea* less virulent than other cork oak leaf pathogens, such as *Elsinoë quercus-ilicis* (Arn.) Jenkins and Goid. and *Phloeospora ilicina* Sacc. *Phomopsis quercella*, which showed moderate pathogenic effects in the stem assay, was clearly pathogenic on leaf regardless of the irrigation treatment. This species was previously cited from cork oak twigs (FRANCESCHINI et al. 1993; MUÑOZ et al. 1996), but also from other *Quercus*, especially *Q. robur* and *Q. petraea* (DA CAMARA 1949; KOWALSKI and KEHR 1992; HALMSCHLAGER et al. 1993; SIEBER et al. 1995). It has been previously reported that *P. quercella* developes an endophytic growth inside the tree and takes advantage of stressed or dying tissues for further colonization (GRIFFITH and BODDY 1990; KOWALSKI and KEHR 1992).

Biscogniauxia mediterranea did not cause any significant leaf lesions, but maintained its viability regardless of the water treatment. *Lembosia quercina* must also be considered a cork oak pathogen in spite of its negative re-isolation. Although foliar lesions caused by *L. quercina* were scarce, small in size and re-isolations did not occur, there is no doubt that inoculation produced the same characteristic symptoms as observed under natural conditions. MARRAS (1963) noticed it on cork oak leaves for the first time in Sardinia. In addition to *Q. suber*, 10 further host species of *Quercus* are cited for *L. quercina* (FARR et al. 1989).

Although the pathogenicity of *Fusarium*, *Phytophthora*, *Pythium* and *Verticillium* species is widely documented, none of the strains used in the root inoculation test showed their pathogenicity clearly. Only *F. oxysporum*, *F. solani* and *P. cinnamomi* caused moderate root lesions and, in general, there were no aerial symptoms except one plant killed by *P. cinnamomi*. The reasons for failure of expression of pathogenicity are unknown but were probably related to the experimental method. In spite of these negative results, stem inoculations with *F. solani* and *P. cinnamomi* have shown the susceptibility of cork oak to these species. On the other hand, *V. tenerum*, which also showed negative results in the root inoculation experiment, was only moderately virulent when inoculated in the stems of water-stressed plants.

In conclusion, 14 fungal species out of the 34 tested proved to be pathogenic; 11 on stem and three on leaf. In addition to the widely known pathogens of cork oak such as *B. mediterranea*, *B. stevensii* and *P. cinnamomi*, *Phomopsis* sp. was particularly virulent on water-stressed plants. In general, trunk pathogens were affected negatively by water stress, as demonstrated by a reduction in both the number and size of the lesions. Nevertheless, *B. mediterranea* and *Phomopsis* sp. increased the number of cankers and length of vascular lesions under stress conditions, whereas *B. stevensii* did not seem to be affected by water restriction. The latter species was the most virulent of all the strains tested. Leaf pathogens caused only local necroses and were influenced negatively by water stress.

Acknowledgements

J.L. was the recipient of a fellowship (FI-PG/94–9.806) provided by the Direcció General de Recerca de la Generalitat de Catalunya, for the years 1994–97. The authors are grateful to the researchers who kindly collaborated in fungal identifications: Dr A. Franceschini and Professor F. Marras (Università degli Studi, Sassari, Italy), Dr F. Gené and Dr J. Guarro (Universitat Rovira i Virgili, Tarragona, Spain), Dr J. De Gruyter (Plantenziektenkundige Dienst, Netherland), Professor T. Kowalski (Akademia Rolnicza, Kraków, Poland), Dr M. C. Muñoz and Dr C. Soldevilla (Universidad Politécnica, Madrid, Spain), Dr H. Nirenberg (Biologische Bundesanstalt für Land und Forstwirtschaft, Berlin, Germany), Professor B. C. Sutton (Wenhaston, Suffolk, UK). We also wish to thank Dr C. Roché for the helpful comments made on a previous draft of this paper.

Résumé

Pouvoir pathogène de champignons isolés de Quercus suber en Catalogne

Le pouvoir pathogène de trente-quatre espèces fongiques isolées de chêne liège en Catalogne (nord-est de l'Espagne) de 1992 à 1995 a été testé par inoculation sur tronc, feuilles et racines. Onze espèces se sont montrées pathogènes sur tronc: *Biscogniauxia mediterranea, Botryosphaeria stevensii, Diatrype* cf. *stigma, Endothia gyrosa, Fusarium solani, Graphium* sp., *Ophiostoma quercus, Phomopsis* sp., *Phytophthora cinnamomi, Sporendocladia bactrospora* et un Coelomycète non identifié. Trois champignons ont eu un effet pathogène sur feuilles: *Dendrophoma myriadae, Lembosia quercina* et *Phomopsis quercella.* Aucun effet clair n'a été détecté chez les inoculations de racines. Les pathogènes de tronc se répartissaient en deux groupes selon leurs effets en inoculation; *B. stevensii, Phomopsis* sp. et *P. cinnamomi* provoquaient la mort des plants et induisaient le formation de grands chancres et des nécroses vasculaires. Les autres espèces pathogènes produisaient aussi des chancres graves et des lésions vasculaires, mais pas de mortalité significative. Un stress hydrique augmentait les lésions provoquées par *B. mediterranea* et *Phomopsis* sp. mais limitait ceux de *P. cinnamomi* et des autres champignons inoculés. Cependant, le stress hydrique n'affectait pas significativement les dégâts par *B. stevensii* qui était la plus agressive des espèces testées. Les pathogènes foliaires n'avaient d'effet que si la cuticule foliaire était préalablement endommagée. *Lembosia quercina* provoquait de petites lésions sombres et *D. myriadea* et *P. quercella* provoquaient de grandes plages nécrotiques chez les plants bien arrosés; les lésions causées par ces deux derniers champignons étaient réduites par le stress hydrique.

Zusammenfassung

Pathogenität von aus Quercus suber in Katalonien (Nordostspanien) isolierten Pilzen

Die Pathogenität von 34 Pilzarten, die im Zeitraum 1992–1995 von Korkeichen (*Quercus suber*) in Katalonien (NO-Spanien) isoliert wurden, wurden mit Hilfe von Trieb-, Blatt- oder Wurzelinokulationen untersucht. Am Stamm erwiesen sich 11 Arten als pathogen: *Biscogniauxia mediterranea, Botryosphaeria stevensii, Diatrype* cf. *stigma, Endothia gyrosa, Fusarium solani, Graphium* sp., *Ophiostoma quercus, Phomopsis* sp., *Phytophthora cinnamomi, Sporendocladia bactrospora* und ein nicht identifizierter Coelomycet. Drei Arten verursachten Symptome auf Blättern: *Dendrophoma myriadea, Lembosia quercina* und *Phomopsis quercella.* Bei den Wurzelinokulationen wurden keine pathogenen Effekte beobachtet. Bei den Stammpathogenen wurden nach den von ihnen an den inokulierten Pflanzen verursachten Symptomen zwei Gruppen unterschieden: *B. stevensii, Phomopsis* sp. und *P. cinnamomi* verursachten den Tod der Pflanzen und induzierten die Bildung von grossen Rinden- und Xylemnekrosen. Die anderen pathogenen Arten verursachten ebenfalls starke Rindennekrosen und Gefässläsionen, es wurde jedoch keine auffallende Mortalität beobachtet. Unter Wasserstress war die durch *B. mediterranea* und *Phomopsis* sp. induzierte Nekrosebildung verstärkt, dagegen war sie bei *P. cinnamomi* und den übrigen inokulierten Pilzen reduziert. Wasserstress beeinflusst jedoch das Ausmass der Schädigung durch *B. stevensii*, der virulentesten der untersuchten Arten, nicht. Die Blattpathogene verursachten nur dann Symptome, wenn zuvor die Blattcuticula beschädigt worden war. *Lembosia quercina* verursachte kleine dunkle Läsionen, während *D. myriadea* und *P. quercella* bei gut bewässerten Pflanzen grosse Nekrosen verursachten. Diese Symptome waren unter Wasserstress weniger stark ausgeprägt.

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