Inoculation of Oak (*Quercus robur* and *Q. rubra*) with *Collybia fusipes*

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ABSTRACT

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Collybia fusipes has been reported as the cause of a root rot disease of oak trees in Europe. The pathogenicity of this fungus has not yet been proven by inoculation. *C. fusipes* was able to survive for more than 1 year in hazel stem segments or on oak root segments buried in nonsterile soil, provided these wood segments were incubated with the fungus for as long as 10 months before burial. We conducted three inoculation trials with 2-year-old oak seedlings. Inoculation success was strongly linked to the ability of *C. fusipes* to survive in the inoculum. The fungus was able to induce substantial lesions (5 to 20 cm^2) in two growing seasons on vigorous seedlings, but none of the inoculated seedlings died in the course of the experiments. Defoliating seedlings for 2 years in a row did not increase the susceptibility to *C. fusipes*.

Collybia fusipes (Bull. ex Fr.) Quel. is a basidiomycetous fungus suspected of inducing a root rot on different species of oak (*Quercus petrea, Q. robur,* and *Q. rubra*). This fungus was only recently reported to be a root pathogen of mature oaks in forest conditions (3,6). Since then, it has been frequently found on pedunculate oak (*Q. robur*) and red oak (*Q. rubra*) in France (Département de Santé des Forêts, *personal communication*). *C. fusipes* induces lesions that have an orange to yellow color with white mycelial fans scattered in the host tissues from which *C. fusipes* can be easily isolated in pure culture. The fungus is sometimes restricted to the bark, but more often it attacks the cambium and rots the wood (3). Buller (2) described perennial structures (pseudorhiza) that develop from roots attacked by *C. fusipes*. We also observed black structures (crusts and cords) on the root surface itself, over typical *C. fusipes* lesions, and also ahead of them, over areas from which the fungus cannot be isolated (*unpublished*). Very little information is presently available on the biology of this apparently common root rot agent. Buller (2) suggested that *C. fusipes* "is parasitic on beeches and oaks, and that its mycelium is able to kill and destroy progressively even their stoutest roots". Nevertheless, no inoculation trials have been performed on oak yet.

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Inoculations of root rotting fungi are generally done by placing a piece of artificially infected wood against the collar of a seedling (4,7–9). The quality of the inoculum is important. Factors such as the nature of the wood piece or the duration of incubation of the wood piece with the fungus before inoculation can influence inoculation success (1,4,9,10; B. Lung, *personal communication*).

Another major factor that can determine the outcome of an inoculation trial is host condition. Some fungi, such *Armillaria gallica*, are not aggressive pathogens and can only induce lesions on hosts that have been previously weakened by another factor (12). A major factor that weakens mature oak trees in European forests is insect defoliation.

Our objective was to confirm through inoculation trials that *C. fusipes* can cause a root rot disease. First, we tried to produce an inoculum on which *C. fusipes* could grow and survive well in soil. We also tested the hypothesis that *C. fusipes* could be a stress-induced pathogen by inoculating defoliated oaks.

MATERIALS AND METHODS

Plant material and fungus isolates. Two-year-old oak seedlings were inoculated. Pedunculate oaks were from the Azeirex provenance (Hautes Pyrénées). Red oaks were from the Saint Florentin provenance (Deux Sevres) in experiment 3 and from the Pierroton provenance (Gironde) in experiment 5. They were transplanted into a mix of an equal volume of sand and forest soil (from Amance forest, Meurthe et Moselle) in experiments 3 and 4 (pots of 9 and 5 liters, respectively), and into a mix of an equal volume of peat and sand in experiment 5 (pots of 5 liters). The seedlings were kept in a glasshouse and were watered daily in the three inoculation experiments (3, 4, and 5). They were fertilized with 12 g of Nutricot per pot (14-8-8, N-P-K) each year in experiment 5. *C. fusipes* strain C49, isolated from an infected red oak in 1992 at les Barres, Loiret, was used for all experiments.

Production of inoculum. Pieces of wood were colonized by *C. fusipes* according to a method adapted from Guillaumin (5). Stems and roots 1.7 to 2.3 cm diameter, with the bark intact, were collected and cut into segments 3 cm long. They were placed in glass jars (18 to 25 per jar) filled with tap water and sterilized twice at 120°C for 30 min 24 h apart. The water was drained at the end of each sterilization. A liquid malt medium was added to half the height of the wood segments, and a third sterilization was done for 20 min at 120°C. To improve aeration, a hole was drilled in the jar top and plugged with cotton wool. Ten blocks of inoculum (0.5 × 0.5 cm) from a *C. fusipes* culture growing on malt agar medium (20 g of malt Difco and 15 g of agar per liter) were aseptically added to the glass jar. The wood segments were incubated for 30 to 45 days at 23°C, then all the liquid was drained from the jars with a syringe and they were further incubated at 23°C for 1 to 9 months.

Survival of *C. fusipes* in woody tissue. In experiment 1, the survival of *C. fusipes* in four woody substrates buried in soil was investigated. The woody substrates were sessile oak (*Q. petrea*) roots and stems and poplar (*Populus* × *euramericana* cv. Robusta) and hazel (*Corylus avellana)* stems. After these inocula were incubated for 4.5 months, they were removed from the jars and placed in 5-liter pots containing the same soil as in experiments 3 and 4 (10 pieces of wood per pot, four pots per wood substrate). The pots were kept in a greenhouse. Two to three wood segments were removed from each pot after 1, 3, 6, and 15 months. They were washed under water and surface sterilized (1 min in sodium hypochlorite at 3.75% active chlorine) and rinsed three times in sterile water. Eight chips from each of the wood segments were then plated on MAT medium (10 g of malt Difco, 100 mg of penicillin, 100 mg of streptomycin, 250 mg of thiabendazole, and 15 g of agar per liter).

In experiment 2, the survival of *C. fusipes* in two woody substrates (hazel stems and red oak roots) buried in two types of soil was investigated. The soils were (1) the same soil as in the previous experiment and (2) a mix of an equal volume of peat and sand. The inoculum was

incubated for 10 months, removed from the jars, and placed in 5-liter pots that were kept in a greenhouse (10 wood segments per pot, two pots per wood substrate per type of soil). Two to three wood segments were removed from each pot after 1, 2.5, 8, and 14 months and treated as in the previous experiment.

Inoculation experiments. In experiment 3, 42 seedlings each of pedunculate and red oak were inoculated at the end of July 1993 with hazel tree stem segments incubated with *C. fusipes* for 2 months. Soil from the base of the seedlings was removed, and the collar area was brushed and washed with water. The inoculum was attached tightly to the collar at 2 to 5 cm under the soil level by a plastic link, and the soil was replaced. Twelve additional control seedlings were inoculated with uncolonized wood segments. Two years after inoculation, at the end of May 1995, seedlings were removed from the pot, and the collar area and inoculum were examined. The condition of the inoculum was recorded: survival of *C. fusipes* was assumed when black crusts covered the inoculum and white mycelium was present underneath or when the wood had a bright orange color. Otherwise, *C. fusipes* was assumed to have disappeared from the inoculum. The collar area was surface sterilized as described in the survival experiments. Outer bark was removed, and chips of dead bark were plated on MAT medium. Width and height of dead areas of bark and cambium were recorded. Inoculation was recorded as successful only when cambial death had occurred. For estimating the surface area affected, lesions were assumed to be circular, with a diameter equal to the mean of the width and the height.

In experiment 4, 100 red oak seedlings were inoculated in mid-June 1994. Collar areas were exposed and washed, and six different inoculation procedures were tested (15 plants per procedure): (1) wounding to the wood with a scalpel, applying an agar block colonized by *C. fusipes*, and wrapping with Parafilm; (2) wounding with a scalpel to the wood, applying a 2.5 cm^3 piece of dry beech wood (autoclaved two times for 20 min at 120°C and incubated for 2 months on *C. fusipes* growing on malt agar plate), and wrapping with Parafilm; (3) same procedure as 2, without wounding; (4) same procedure as in experiment 3, with hazel stem segments incubated for 10 months; (5) same procedure as 4, but with red oak root segments incubated for 10 months; (6) same procedure as 4, but with red oak root segments incubated for 6 months. Five additional control oaks were inoculated according to procedures 2 and 4 with uncolonized wood segments. In September 1995, 15 months after inoculation, seedlings were removed from the pots, and the inoculum and the collar area were examined and treated as in experiment 3. In this experiment, the presence of *C. fusipes* crust on the surface of the host roots was recorded.

In experiment 5, 20 pedunculate oak seedlings were inoculated in early March 1994 with hazel stem inoculum incubated for 10 months. Thirteen additional control trees had uncolonized hazel stem segments attached to the root collar. Ten each of inoculated and control seedlings were completely defoliated on 4 August 1993, and again on 4 August 1994. Leaves were removed by cutting the petiole. The growth of the first and second terminal flush were measured both in 1994 and 1995. In August 1995, 18 months after inoculation, seedlings were dug out, and the collar area was sampled and treated as in experiment 3.

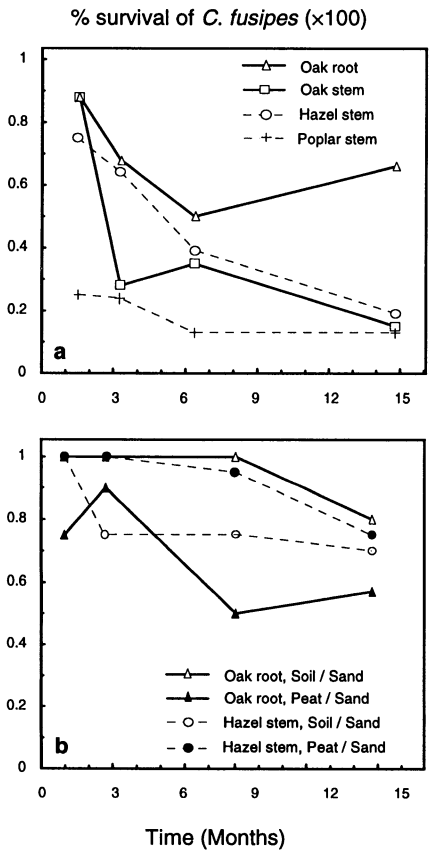

Fig. 1. Survival of *Collybia fusipes* on several kinds of wood segments buried in soil: **(A)** experiment 1, and **(B)** experiment 2. Wood segments were incubated 4.5 months in experiment 1 and 10 months in experiment 2 before being buried in soil.

RESULTS

Survival of *C. fusipes* in woody inoculum. There were large differences between experiments 1 and 2 in the survival of *C. fusipes* in the wood segments. In experiment 1, with a 4.5-month precolonization of the segments, recovery of *C. fusipes* from stem segments of oak, hazel, and poplar decreased quickly during the first 6 months (Fig. 1). At the end of the experiment (15 months), *C. fusipes* was recovered from only 20% of all stem segments. The fungus survived best on oak root segments, and at the end of the experiment, it was recovered from 60% of those segments. In experiment 2, with a 10-month precolonization of the segments, recovery from the two types of wood substrate, hazel stem and oak root, was high and remained stable during 14 months in soil. At the end of the experiment, *C. fusipes* was recovered from 60 to 80% of both substrates. There was no significant difference in the survival of the fungus in the soil:sand mix or in the peat:soil mix (Fig. 1).

Inoculation experiments. In experiment 3, survival of *C. fusipes* in the wood segments was low. Only eight out of 84 wood segments, i.e., 10%, were still colonized by the fungus at the end of the experiment (two on red oaks and six on pedunculate oaks). No lesions were seen on oaks where *C. fusipes* was no longer present in the wood segment at the end of the experiment. Infection was always successful when *C. fusipes* was still present in the wood segment. On infected seedlings, the areas of bark lesion and of cambium death averaged 24.3 ± 6.8 cm^2 and 16.7 ± 7.5 cm^2, respectively. *C. fusipes* was reisolated from four of the eight seedlings. The average diameter of the seedling collar was 2.8 cm.

In experiment 4, infections were more frequent with hazel stem and oak root inocula incubated for 10 months (47 to 53%) than with all other types of inocula (0 to

Table 1. Comparison of several procedures to inoculate 2-year-old *Quercus rubra* seedlings with *Collybia fusipes*

Inoculation treatment/ inoculum substrate	Number of seedlings: Total	Inoculum survival[w]	Seedlings with lesions	Size bark lesion (cm^2)[x]	Size cambial lesion (cm^2)[x]
W[y]/agar	15	0	0	–	–
W/beech wood (2)[z]	15	8	0	–	–
N/beech wood (2)	15	6	1	2.0	2.0
N/oak root (6)	15	9	2	1.8	1.1
N/oak root (10)	15	15	8	1.3 ± 0.3	0.7 ± 0.4
N/hazel stem (10)	15	15	7	4.9 ± 2.5	3.5 ± 2.6

[w] Number of seedling for which *C. fusipes* was still present on wood segments at end of experiment.
[x] For successful infections only. Mean ± standard error of the mean.
[y] W = wounded, N = nonwounded.
[z] Time in months of incubation of wood segments before inoculation is given in parentheses.

13.4%, Table 1). In the case of beech wood inocula, wounding the bark did not significantly influence the infection. The outcome of the inoculation was strongly dependent on the survival of *C. fusipes* in the wood segments. The inoculation procedures that were most successful were those using wood segments in which *C. fusipes* survived well (Table 1). The fungus survived in only 40 to 60% of the beech wood pieces and oak root segments, respectively, incubated for 2 and 6 months prior to inoculation. Fungal survival was 100% in hazel stem and oak root segments incubated for 10 months prior to inoculation. For successful infections, the surface area of both bark and cambium lesions was significantly greater with hazel stem segments than with oak root segments (Student's *t* test of 2.52 and 2.28, respectively, df = 11, Table 1). *C. fusipes* crusts could be seen on the surface of only five oak collars, all of which were inoculated with hazel stem segments incubated for 10 months. The average diameter of the seedling collar was 1.2 cm.

In experiment 5, defoliated seedlings refoliated poorly in August and September 1993, after the first defoliation. They did not refoliate at all after the 1994 defoliation. The first defoliation had no significant influence on the total shoot growth of the seedlings in 1994 (Table 2). However, the first shoot flush of defoliated oaks was reduced in 1994 (Table 3). After 2 years of defoliation, stressed oaks had a significantly reduced growth in 1995 (Tables 2 and 3). Inoculation alone did not decrease the growth of the trees in 1994, but the inoculated and nondefoliated trees grew less than the controls in 1995, which explains the significant inoculation × defoliation interaction that year (Tables 2 and 3). All oaks, including defoliated and/or inoculated seedlings, had a good crown condition at the end of the experiment. All inoculated seedlings were infected, and *C. fusipes* was still present on all wood segments at the end of the experiment. Typical orange lesions with white mycelial fans were present on all inoculated seedlings. The lesions extended mainly tangentially, often girdling the collar at the bark level, but they usually had a poor longitudinal extension. As a result, infected seedlings were able to grow new root from the collar above the lesion. For 95% of them, *C. fusipes* could be reisolated from infected tissues. *C. fusipes* black crusts were present on the collars of 90% of the inoculated seedlings. Crusts were also present over areas of necrotic bark from which the fungus could not be reisolated. Defoliated seedlings had significantly smaller bark lesions than did the nondefoliated seedlings (Student's *t* test of 3.28, df = 18, Table 3) but similar-sized cambial lesions (Student's *t* test of 0.74, df = 18). The average diameter of the seedling collar was 2.6 cm.

DISCUSSION

C. fusipes successfully infected pedunculate and red oak seedlings. Lesions were similar to those that develop on mature trees in natural conditions. In particular, *C. fusipes* crusts were observed on the root surfaces of inoculated seedlings in advance of the typical orange lesion from which the fungus can be reisolated. This suggests that *C. fusipes* can move along the bark tissues using these structures. Such crusts are common among root rotting basidiomycetes and are often referred to as ectotrophic mycelia (9,10).

Successful infection was strongly linked to the survival of *C. fusipes* on the wood segments. Whatever the experiment, this fungus survived well when wood segments were incubated for at least 10 months before inoculation (75 to 100%, Table 1, Fig. 1B). When the wood segments were incubated for shorter periods of 2 to 6 months, survival was much lower (10 to 60%). *C. fusipes* survived equally well on hazel stems and on oak roots incubated for 10 months before inoculation (Fig. 1B, Table 1). In experiment 4, infection success was comparable with those two types of inocula, but the size of the lesions induced was larger with the hazel stem segments than with the oak root segments (Table 1). Thus, in this study, hazel stem proved to be a superior substrate to prepare *C. fusipes* inoculum. Moreover, hazel stem is far easier to collect than oak roots.

In experiment 5, young oaks stressed by defoliation during two consecutive summers seemed to be less susceptible than the nondefoliated controls, having the same amount of cambial death but bark lesions about two times smaller than the latter (Table 3). Perhaps the defoliated trees were not weakened enough. The timing of the defoliation, in early August, was probably too late in the summer to cause a major stress. Young white and black oaks were more seriously weakened when defoliated in July than in August in the northeastern United States (11). Moreover, Wargo and Houston (13) showed that the susceptibility of sugar maple to *Armillaria mellea sensu lato* was enhanced much more by a spring defoliation than by an August defoliation. In both cases, defoliation weakened the trees most severely if they refoliated at the end of the summer, which happened poorly in our study. We therefore cannot draw conclusions on the influence of defoliation on the susceptibility of pedunculate oak to *C. fusipes*. Moreover, in Europe, most of the oak defoliation by insects occurs in spring.

In the three trials we conducted, *C. fusipes* attacked vigorous young oaks. The lesions induced in two growing seasons were nevertheless of a relatively limited size (5 to 20 cm^2), and none of the inoculated oaks died or showed dieback. Therefore, this fungus is not a very aggressive pathogen. This conclusion corresponds well with what we know of the fungus from field observations. However, this work shows that *C. fusipes* can colonize healthy roots and should be considered a primary organism able to induce serious lesions on healthy oaks.

Table 2. Analysis of variance of the effect of defoliation and *Collybia fusipes* infection on shoot growth of young *Quercus robur*

Year Source	df	Mean square	*F*	*P*
1994				
Model	3	1,866.6	2.70	0.064
Error	29	692.5		
1995				
Model	3	2,788.1	3.58	0.026
Error	29	779.3		
Inoculation effects	1	2,332.3	2.99	0.094
Defoliation effects	1	7,437.7	9.54	0.004
Defoliation × inoculation effects	1	3,404.3	4.37	0.046

Table 3. Interaction of defoliation and *Collybia fusipes* infection of young *Quercus robur* on shoot growth and lesion size

		Shoot growth (cm)		Lesion size (cm^2)	
Treatment	No.	1994[y]	1995[y]	Bark	Cambium
Control	3	60.2 ± 31.1 a (24.2)	96.3 ± 21.4 a (30.7)	–	–
Inoculated	10	70.8 ± 19.7 a (22.5)	53.9 ±1 6.7 b (17.3)	22.6 ± 4.2	8.8 ± 4.1
Defoliated[z]	10	45.5 ± 16.6 a (10.4)	38.8 ± 16.7 b (4.3)	–	–
Inoc. & defol.	10	45.5 ± 9.0 a (9.7)	42.8 ± 19.2 b (4.4)	11.4 ± 4.2	6.7 ± 3.9

[y] Length in cm of 1994 and 1995 first flush is given in parentheses. Mean ± standard error of the mean. Treatments followed by a different letter are significantly different for scheffe test at the 0.05 level.

[z] In August 1993 and 1994.

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