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Fungi associated with esca disease in grapevine

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Abstract

Cross sections of woody stems of 309 diseased grapevines collected in France showed two kinds of necrosis typical of esca: a) A central light-colored necrosis of soft consistency, consisting of three zones, preceded by a centrally discolored wood, and b) a sectorial light-colored necrosis composed of two zones preceded by a sectorial brown necrosis. Isolations showed that different microflora was associated with each necrosis. *Phaeoacremonium aleophilum* and *Phaeoacremonium chlamydosporum* occurred in the discolored wood and the zones bordering the central decayed wood. *Eutypa lata* was the main fungus isolated from sectorial brown necrosis and the zones adjoining the decay wood. *Phellinus punctatus* was isolated from the sectorial and central decayed wood. *Stereum hirsutum* was present in decayed wood of 15 grapevines with esca symptoms not inhabited by *P. punctatus*. Wood decay tests and pathogenicity tests showed that *S. hirsutum* and *P. punctatus* were responsible for the decayed wood. *Phaeoacremonium chlamydosporum* and *S. hirsutum* produced a centrally discolored wood similar to that found in esca-affected vines. *Phaeoacremonium aleophilum* caused a sectorial brown necrosis of soft texture. From these studies, it was found that esca is a complex disease involving several microorganisms whose role in the process leading to wood degradation is discussed.

Introduction

Esca is one of the most destructive diseases of the woody tissues of grapevine (*Vitis vinifera* L.) in Europe and California, where it is called black measles (Chiarappa, 1959; Dubos and Larignon, 1988). Currently, this disease is less important in France than in other Mediterranean countries. It can be controlled by applications of sodium arsenite during the winter dormancy.

Symptoms can appear in severe or mild forms (Viala, 1926; Arnaud and Arnaud, 1931; Chiarappa, 1959; Baldacci et al., 1962; Branas, 1974; Galet, 1977; Dubos and Larignon, 1988). The mild form is characterized by foliage deterioration. The leaves have interveinal islands of chlorotic and yellowish tissue, which later becomes necrotic. Grape clusters appear normal, but berries do not fill properly and generally do not reach maturity. Another type of the mild form has been observed in two vineyards in Italy (Graniti, 1960; Grasso, 1969) and California (Chiarappa, 1959); this type is characterized by dark, purple spots scattered over the epidermis of the berries which may remain turgid until maturity or crack and dry. These symptoms may occur without symptoms on leaves. The severe form of the disease, called apoplexy, is characterized by the sudden wilting and death of bearing vines or vine-parts in midsummer. A cross section of the wood of affected trunks or arms of different forms shows a central necrotic and decayed area surrounded by a black line (Chiarappa, 1959; Baldacci et al., 1962; Grasso, 1969; Galet, 1977).

Before conducting experiments to find an alternative control method as effective as sodium arsenite, it was necessary to know more about the microorganisms implicated in this disease. An attempt was made to correlate the microorganisms with the types of discoloration and decay in woody tissue of grapevines; the pathogenicity of the most frequently isolated fungi was tested using both inoculated wood blocks and

inoculated living grape stems. This paper contributes knowledge towards determining the cause on grapevines and is thus a step in understanding the disease.

Materials and methods

Isolations

309 diseased grapevines of different cultivars (Aubun, Cabernet Sauvignon, Carignan, Cinsault, Grenache, Mourvèdre, Pinot meunier, Sauvignon, Tempranillo, Ugni blanc) were obtained from various viticultural districts of France (Armagnac, Bergerac, Bordeaux, Bourgogne, Champagne, Cognac, Corse, Côtes-du-Rhône and Languedoc-Roussillon) between 1985 and 1988. All vines were 5–30 years old and had external disease symptoms on the foliage (mild or severe forms). Cross and longitudinal sections of the woody stem of each vine were examined in order to follow development of necrosis in the trunks, and the type of necrosis was recorded.

After wood collection, isolations were made from different zones of necrotic tissue. Small pieces of tissue from firm wood were submerged in 30% calcium hypochlorite for 15 sec. Samples from soft, spongy wood were rapidly passed over a flame. Twenty tissue pieces, approximately $3 \times 1 \times 1$ mm in size, from each zone were placed in plastic petri dishes containing malt agar medium (15 g cristomalt, 20 g agar per 1000 ml of water) and incubated in the dark at room temperature (20–25 °C). Observations were recorded at weekly intervals.

Basidiocarps of *S. hirsutum* and *P. punctatus* were collected in the vineyards or were artificially produced by incubating grapevine trunks in a moist chamber. The main fungi isolated from wood were identified with the references cited: *Acremonium* (Gams, 1971), *Phaeoacremonium* (Crous et al., 1996), *Phellinus* (Stalpers, 1978; Larsen and Cobb-Poule, 1991), *Phialophora* (Schol-Schwarz, 1970; Cole and Kendrick, 1973; Hawksworth and Gibson, 1976), *Stereum* (Welden, 1971; Stalpers, 1978; Chamuris, 1988), *Eutypa* (Carter, 1991; Rappaz, 1987), *Botryosphaeria* (Shoemaker, 1964; Lehoczky, 1988).

Results were given as a frequency of necrotic pieces infected with each microorganism for each zone of the necrosis and as a frequency of discs infected with each microorganism (T value). All the fungi isolated at low frequency (<1%) were grouped under the name ‘other fungi’.

Fungal isolates

Phaeoacremonium aleophilum W. Gams, Crous, M. J. Wingf. et L. Mugnai, sp. nov. (Muséum d’Histoire Naturelle de Paris, LCP 93 2940), *Phaeoacremonium chlamydosporum* W. Gams, Crous, M. J. Wingf. et L. Mugnai, sp. nov. (LCP 93 2941), *Phellinus punctatus* (Fr.: Karst.) Pilat in Kavin et Pilat (LCP 93 2942), and *Stereum hirsutum* (Willd.: Fr.) S. F. Gray (LCP 93 2975) were isolated from diseased vines collected in 1985 in Bordeaux vineyards. They were tested for pathogenicity with young vines and in decay tests with blocks of healthy grapewood. Each fungus was grown on malt agar in petri dishes and tubes at 22 °C in the dark.

Pathogenicity tests

One-year-old canes of Cabernet Sauvignon 1 cm in diameter were cut into one-node segments each 10 cm long, disinfected by dipping in a 30% calcium hypochlorite solution for 15 min and rinsed in sterile tap water. A few millimeters of tissue was removed from the ends of segments to eliminate tissue injured by hypochlorite. A hole 3 mm in diameter was drilled 0.5 cm below the bud until the pith. The hole was filled with either 20 μl of mycelial suspension prepared by blending a petri dish culture of the test fungus (*S. hirsutum*, *P. punctatus*) in sterile distilled water or 20 μl of spore suspension of *P. aleophilum* or *P. chlamydosporum* (approximately 10^3 conidia/ml). Control canes were filled with sterile distilled water. Filled holes were sealed with handwarm molten paraffin.

For each fungal isolate, 30 cane segments were used; they were kept for approximately 2 months in plastic trays filled with sand to promote sprouting and rooting, after which they were planted individually in small plastic pots containing a compost and sand mixture (3:1). After a further four months in the greenhouse (temperature = 23±2 °C, light/dark = 16/8 h), the wood of 20 plants was examined in the laboratory for disease symptoms. Ten other plants were then left outside for another year. The plants had been inoculated in March 1986; the greenhouse plants were examined in August 1986 and the outdoor plants in September 1987. The plants were cut across and longitudinally and twenty necrotic pieces of wood per plant were plated onto malt extract agar as previously described.

Woody decay tests

The ability of each fungus to produce wood rot was determined by inoculating wooden blocks obtained from healthy trunks of Cabernet Sauvignon under sterile conditions in the laboratory. Blocks of healthy wood, $10 \times 5 \times 30$ mm in size, were sterilized by autoclaving for 20 min at 120 °C. Blocks were soaked in sterile distilled water for 10 min and placed in a culture tube containing the test fungus grown on malt agar medium. Ten blocks were used for each fungus. Blocks were incubated in the dark at 25 °C. After 12 months, the blocks were examined for rot.

Results

Description of necrotic wood in the trunk

The 309 diseased vines were classified into five categories I, II, III, IV and V, on the basis of decay in the main trunk (Figure 1):

- Category I (145 vines) had a central area of light-colored necrosis of soft consistency composed of three zones (Figure 1A). A central zone characterized by light color and soft texture (Figure 1A-a) bordered throughout by a brownish dark zone of irregular width and outline (Figure 1A-b) and separated from the healthy wood by a pinkish brown zone of hard consistency (Figure 1A-c). The necrosis extended throughout the length of the trunk.
- Category II (31 vines) showed discolored wood only in the center of the trunk (Figure 1B). This necrosis was characterized by a brown zone of hard consistency (Figure 1B-f), and a pinkish brown zone of hard consistency (Figure 1B-c).
- Category III (64 vines) showed two necroses all over the trunk, a central, light-colored necrosis of soft consistency (Figure 1C-a) which was preceded by centrally discolored wood (Figure 1C-c, f), the same as described above (categories I and II).
- Category IV (54 vines) showed two necroses in the same cross section (Figure 1D), one necrotic area similar to the light-colored necrosis of soft consistency (Figure 1D-a, b, c) described in category I, and a second brown and firm necrotic area in sectorial position (Figure 1D-a, e, d). A light, soft zone (Figure 1D-a) was delimited by a brown zone (Figure 1D-e, d). These necroses extended over the whole length of the plant.
- In category V (15 vines), the two light-colored necroses of soft consistency previously described (category IV) were combined in the same cross section, and preceded by a centrally discolored wood (Figure 1E-c, f) and a sectorial brown necrosis (Figure 1E-e, d).

Our observations clearly show that the necroses of all five categories originated at pruning wounds.

Isolations

The fungi most frequently isolated from the central light-colored necrosis observed in diseased trunks of categories I (Figure 1A) and III were *P. aleophilum*, *P. chlamydosporum*, and *P. punctatus* (Table 1). They were encountered in almost all disks examined. They were isolated from different areas of the necrosis. *P. aleophilum* and *P. chlamydosporum* were more frequently isolated from the discolored zones surrounding the decayed areas (Figure 1A-b, c) and seldom from the light zone of soft texture (Figure 1A-a). *P. punctatus* did not commonly occur in the hard discolored zones (Figure 1A-b, c), but was frequently isolated from the soft decayed areas (Figure 1A-a).

In diseased trunks of categories II and III (Table 2), *P. aleophilum* and *P. chlamydosporum* were the fungi most commonly isolated from the pinkish-brown zone (Figure 1B-c) and the brown zone (Figure IB-f).

In the trunks of categories IV (Figure 1D) and V, the fungi most frequently isolated from central and sectorial light-colored necroses of soft texture were *E. lata*, *P. punctatus*, *P. aleophilum* and *P. chlamydosporum*. They were isolated from 100%, 78.2%, 98.6%, and 91.3% of examined disks respectively. *E. lata* was frequently isolated from sectorial zones surrounding the decayed wood (Figure 1D-d, e) (Table 3). *P. aleophilum* and *P. chlamydosporum* were present in high frequencies in the discolored zones bordering decayed wood (Figure 1D-b, c). *P. punctatus* occurred most frequently in decayed wood (Figure 1D-a).

In the trunks of category V, the four fungi isolated most frequently from centrally discolored wood and sectorial brown necrosis were *E. lata*, *P. punctatus*, *P. aleophilum* and *P. chlamydosporum* (Table 4). These fungi were isolated from almost all disks examined, but located in different areas of the necrosis. Discolored wood in the center (Figure 1E-f, c) commonly yielded *P. aleophilum* and *P. chlamydosporum*, and *E. lata* was mostly absent (Figure 1E-f, c). It occurred in the brown necrosis located sectorially (Figure 1E-e, d). Except in

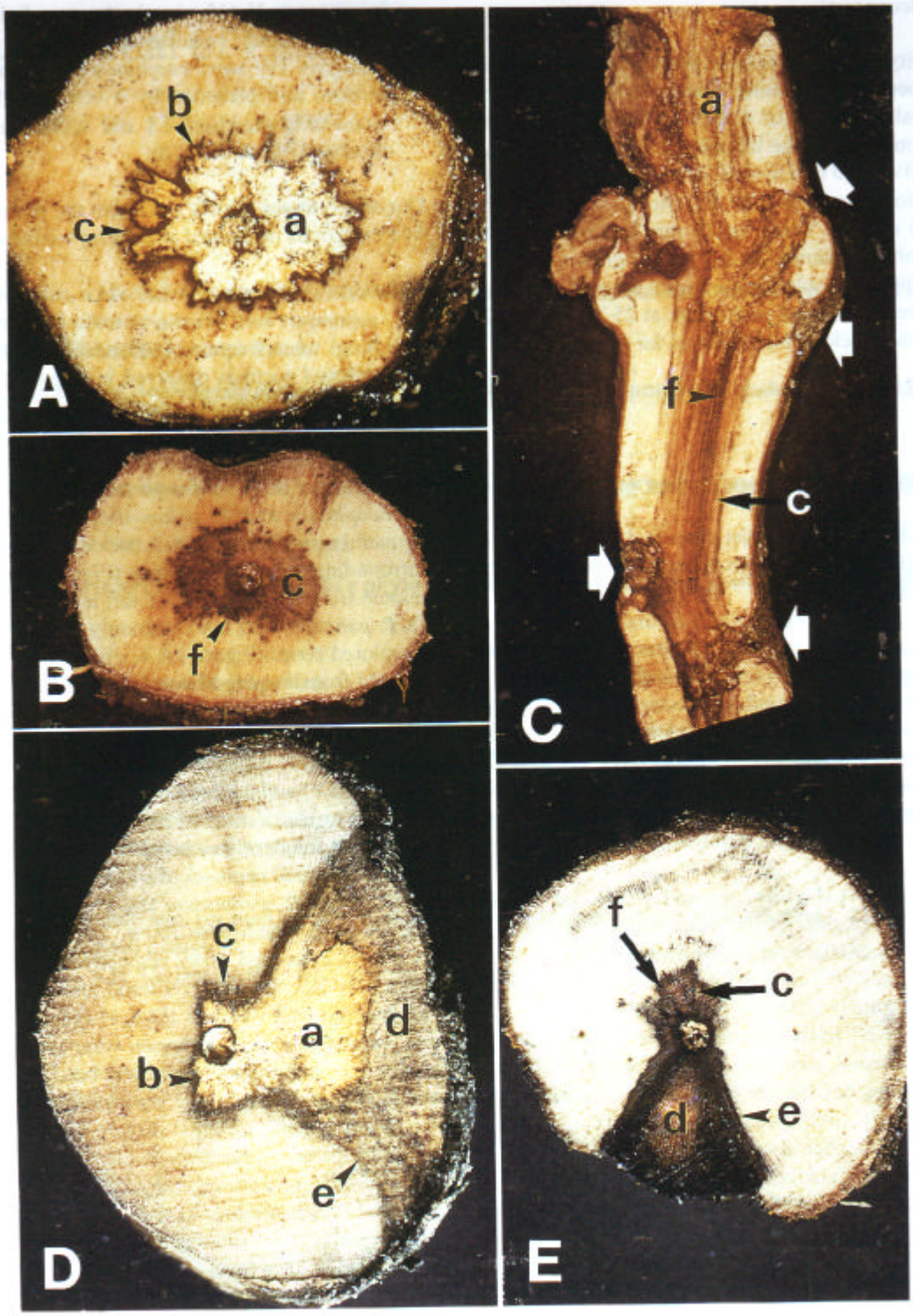
A
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Table 1. Identity and frequency of organisms isolated from centrally light-colored necrosis of soft texture (categories I and III),[1] zones a, b, c as shown in Figure 1A, h = healthy wood

Organisms isolated	Frequency (%)				
	a[2]	b[2]	c[2]	h[2]	T
Phaeoacremonium chlamydosporum	2.2	31.6	23.6	0.0	96.2
Phaeoacremonium aleophilum	0.7	22.8	25.7	0.0	88.5
Phellinus punctatus	33.3	2.4	4.1	0.0	83.7
Botryosphaeria obtusa	13.3	3.3	6.7	0.0	73.7
Penicillium sp.	5.2	1.1	1.9	0.0	45.5
Alternaria sp.	2.7	0.3	5.0	0.0	37.8
Gliocladium sp.	0.6	1.8	5.5	0.0	28.2
Aspergillus sp.	4.4	1.7	3.9	0.0	26.3
Pestalotia sp.	0.8	0.8	2.6	0.0	22.0
Phomopsis sp.	0.0	2.7	3.5	0.0	8.6
Stereum hirsutum	5.0	0.6	0.2	0.0	8.1
Mycelia sterilia	4.9	2.0	1.3	0.0	14.8
Other fungi	14.9	2.2	5.7	0.0	–
Bacteria	2.6	2.3	3.2	0.0	74.6
Total	90.6	75.6	92.9	0.0	
Sterile pieces	13.8	30.0	10.5	100.0	
Total	104.4	105.6	103.4	100.0	

[1] Number of vines examined: 209.
[2] Based on 4180 pieces of tissue for each decay type used for isolation.

categories II and III, no microorganisms were isolated from healthy wood.

Stereum hirsutum was commonly isolated from decayed wood (Figure 1A-a, 1C-a) in several types of diseased trunks. Tables 5 and 6 show the microflora accompanying *S. hirsutum* in the trunks of categories I and III. *S. hirsutum* did not occur in trunks colonized by *P. punctatus*. It was present in the discolored zones bordering decayed wood and discolored zones of the brown necrosis (Figure 1C-c, f). The microflora observed was almost the same as that accompanying *P. punctatus*. Two principal fungi were encountered. *P. aleophilum* and *P. chlamydosporum* were found in high frequencies in the zones bordering decayed wood (Figure 1A-b, c) and in the discolored wood (Figure 1B-c, f). *Stereum hirsutum* was never isolated from healthy wood.

Morphological characteristics of isolates

Cultures of *P. aleophilum* (LCP 93 2940) on malt agar slow-growing, reaching 1–2 cm in diameter in the dark after 8 days at 30 °C (temperature optimum), greyish white at first, becoming olive-brown; mycelium slightly raised; reverse greyish brown to dark brown. Aerial mycelium greyish brown. After 8–15 days in culture, colonies producing a diffusing yellow pigment in the medium. Hyphae thin-walled, hyaline to brownish, 1.6–2 μm wide, occasionally compacted in strands, sometimes becoming deeply pigmented. Sporulation abundant. Conidiophores, frequently reduced to conidiogenous cells, rarely 1–2 septate, brown in the lower part, becoming lighter towards the tip. Conidiogenous cells phialidic, mostly solitary, occasionally on sparsely branched conidiophores, variable in length,

Figure 1. Cross sections (A, B, D, E) and longitudinal sections (C) of trunks associated with typical esca symptoms on herbaceous parts. A, light-colored necrosis of soft consistency in the center surrounded by a black zone (category I); B, discolored wood in the center (category II); C, central wood decay preceded by central discolored wood. Arrows indicate probable points of entry of wood rotting organisms (category III); D, light necroses of soft consistency in sectorial and central position (category IV); E, central discolored wood and sectorial brown necrosis of hard consistency (category V).

Abbreviations: a = white decay of soft texture; b = black line; c = pinkish brown zone of hard consistency; d = sectorial brown zone of hard consistency; e = edge of sectorial brown zone; f = brownish dark zone of hard consistency.

Table 2. Identity and frequency of organisms isolated from centrally discolored wood (Categories II and III),[1] zones f, c as shown in Figure 1B, h = healthy wood

Organisms isolated	Frequency (%)			
	f[2]	c[2]	h[2]	T
Phaeoacremonium aleophilum	20.7	16.6	0.1	87.4
Phaeoacremonium chlamydosporum	20.2	8.6	0.5	85.3
Botryosphaeria obtusa	10.5	4.0	0.6	58.9
Alternaria sp.	3.1	0.8	0.4	42.1
Penicillium sp.	3.4	1.2	0.6	35.8
Gliocladium sp.	4.3	1.2	0.0	31.6
Aspergillus sp.	3.3	1.0	0.0	31.6
Pestalotia sp.	1.6	0.7	0.0	24.2
Rhizopus sp.	0.8	0.5	0.0	9.5
Phomopsis sp.	2.0	0.8	0.0	7.4
Phellinus punctatus	0.8	0.0	0.0	6.3
Stereum hirsutum	0.9	0.2	0.0	2.1
Mycelia sterilia	0.9	0.7	0.0	8.4
Other fungi	7.2	3.1	1.2	–
Bacteria	5.6	3.3	1.4	57.9
Total	85.3	42.7	4.8	
Sterile pieces	23.2	62.7	95.2	
Total	108.5	105.4	100.0	

[1] Number of vines examined: 95.
[2] Based on 1900 pieces of tissue for each decay type used for isolation.

10–30 μm, 2 μm wide at the base, narrowing to 1 μm at the apex. Conidia formed at the apices of the conidiogenous cells, becoming aggregated into dense slimy heads, usually simple, distinctly biguttulate, hyaline, smooth-walled, ellipsoidal to cylindrical with rounded ends, straight or sometimes slightly curved, 4–5.5$\times$1.6–2 μm. Chlamydospores absent.

Cultures of *P. chlamydosporum* (LCP 93 2941) on malt agar slow growing, reaching 7–8 mm in diameter in the dark after 8 days at 25 °C (temperature optimum). Colonies white at first but becoming light green and later dark green, mycelium appressed to slightly raised; reverse brown to dark brown. Hyphae hyaline to pale greenish, occasionally compacted into strands, sometimes becoming deeply pigmented and coarsely ornamented in older cultures, mainly 2–2.5 μm wide. Sporulation abundant. Pigmented conidiophores straight, simple or 1-(2)-branched, sometimes encrusted with a coarse ornamentation, very variable in length. Conidiogenous cells originating singly on the conidiophores, slightly pigmented, subcylindrical to elongate-ampulliform, tending to be slightly inflated below, with a distinct apical narrow funnel-shaped collarette, 15–28 μm long, generally 2–5 μm wide, but narrowing to 0.5–1 μm at the apex; collarette 0.5–2.5 μm long. Conidia formed at the apices of the conidiogenous cells, becoming aggregated into dense slimy heads, non-septate, hyaline, smooth walled, cylindrical, often distinctly 2-guttulate, 2.4–6$\times$1–2 μm. Chlamydospores intercalary, usually formed singly, present particularly in one-month-old cultures.

Cultures of *P. punctatus* on malt agar reaching 4–5 cm in diameter in 2 weeks at 22 °C, white at first, becoming yellow to ochraceous; advancing zone remaining white, smooth, appressed to raised; texture of the mat compact, felty. Reverse showing a few brown lines. Odor absent; mycelium turning brown with KOH. Hyaline hyphae varying in width from 1.5 μm to 4 μm; septa rare and inconspicuous lacking clamp connections; brown hyphae unbranched, straight, varying in diameter from 1.5 to 4 μm, lacking septa.

Basidiocarps perennial, resupinate, becoming widely effused, woody, not readily separable, up to 1 cm thick and 0.5–2.5$\times$1–6 cm in size. Margin yellowish brown at first, becoming ferruginous in

Table 3. Identity and frequency of organisms isolated from centrally and sectorial light-colored necroses of soft texture (categories IV and V),[1] zones a, b, c, d, e as shown in Figure 1D, h = healthy wood

Organisms isolated	Frequency (%)						
	a[2]	b[2]	c[2]	d[2]	e[2]	h[2]	T
Eutypa lata	0.0	2.9	1.1	40.1	23.6	0.0	100.0
Phaeoacremonium chlamydosporum	2.2	20.7	22.0	2.3	1.0	0.0	98.6
Phaeoacremonium aleophilum	0.1	17.6	25.5	1.2	0.9	0.0	91.3
Botryosphaeria obtusa	8.4	2.2	3.9	13.9	9.3	0.0	85.5
Phellinus punctatus	38.6	2.0	0.7	13.6	0.0	0.0	78.2
Epicoccum sp.	3.2	0.4	0.6	4.0	0.0	0.0	46.4
Aspergillus sp.	2.3	1.4	3.2	2.8	1.5	0.0	30.4
Pestalotia sp.	1.6	0.4	1.4	1.3	2.5	0.0	27.5
Penicillium sp.	1.2	1.0	3.6	0.9	2.7	0.0	26.1
Phomopsis sp.	0.0	4.7	4.2	0.0	2.5	0.0	13.0
Alternaria sp.	1.5	0.5	0.4	0.7	0.4	0.0	11.6
Gliocladium sp.	0.4	1.5	3.4	0.4	2.2	0.0	10.1
Cladosporium sp.	1.3	0.5	1.6	3.7	1.5	0.0	0.9
Stereum hirsutum	8.5	1.6	0.1	1.9	0.0	0.0	0.5
Mycelia sterilia	4.3	2.3	2.7	1.7	4.1	0.0	6.0
Other fungi	26.5	5.4	8.1	9.3	3.9	0.0	–
Bacteria	4.9	3.6	4.8	2.3	7.8	0.0	49.3
Total	105.0	68.7	89.3	100.1	63.9	0.0	
Sterile pieces	14.6	38.0	14.6	3.7	1.2	100.0	
Total	119.6	106.7	103.9	103.8	40.1	100.0	

[1] Number of vines examined: 69.
[2] Based on 1380 pieces of tissue for each decay type used for isolation.

older specimens. Pore surface yellowish- to grayish brown; dull, smooth. Pores 6–8 per mm, circular. Contextual skeletal hyphae turning dark brown in KOH, thin- to thick-walled, rarely branched, occasionally septate, 2.5–5 μm wide; contextual generative hyphae almost hyaline and thinner-walled. Setae lacking. Thin-walled, ventricose cystidioid elements present, 20–60 μm long. Basidia 5.7–7.8$\times$2–4 μm. Basidiospores globose, thick-walled, hyaline, smooth, 5–6 μm in diameter.

Cultures of *S. hirsutum* on malt agar growing rapidly, reaching 5–6 cm in diameter in 1 week at 22 °C, white at first, becoming cream to light ochraceous-buff. Advancing zone indistinguishable from the remaining mycelium. Mat immediately behind the advancing zone dense and cottony, later often felty. Reverse bleached. Odor absent. Hyaline hyphae remaining white with KOH; the yellowish parts turning yellowish brown with KOH. Marginal white hyphae varying in width from 1.5 to 8 μm. Clamps lacking at most septa, occurring only on larger hyphae, 8 μm in width, and often in whorls of 4–(6) clamps. Aerial hyphae 1–10 μm wide, sometimes with resinous contents. Coiled hyphae present.

Basidiocarps coriaceous, effuso-reflexed and gregarious varying in size from 0.5 to 1.5 cm. Upper surface of the pileus smoke-grey, tomentose. Hymenial surface brownish orange. Cutis brown. Pseudocystidia cylindrical, thick-walled at the base, thin-walled apically, 4–12 μm in diameter. Hyphidia acuminate, thin- to slightly thick-walled, 2–3 μm in diameter. Basidia narrowly clavate to subcylindrical, 4-sterigmate, 25–40$\times$4–5 μm. Basidiospores hyaline, ellipsoid to cylindrical, 5–7.5$\times$2.5–3 μm.

Pathogenicity tests

P. aleophilum produced a brown necrosis of soft texture located in sectors (Table 7). *P. chlamydosporum* caused brownish dark necrosis on young vines similar to discolored wood on standing vines. Discoloration of wood remained limited to woody tissues present at

Table 4. Identity and frequency of organisms isolated from centrally discolored wood and sectorial brown necrosis (Category V),[1] zones f, c, d, e as shown in Figure 1E, h = healthy wood

Organisms isolated	Brown necrosis					
	Frequency (%)					
	f[2]	c[2]	d[2]	e[2]	h[2]	T
Eutypa lata	3.7	0.0	42.7	13.7	0.0	100.0
Phaeoacremonium chlamydosporum	14.3	17.0	3.7	3.0	0.0	100.0
Phaeoacremonium aleophilum	17.7	17.0	0.0	0.0	0.0	86.7
Botryosphaeria obtusa	10.7	0.7	13.7	3.3	0.0	66.6
Pestalotia sp.	2.7	1.0	0.3	2.0	0.0	46.7
Alternaria sp.	2.0	1.3	2.7	1.3	0.0	40.0
Penicillium sp.	4.0	0.7	2.7	0.7	0.0	40.0
Aspergillus sp.	1.0	0.7	1.0	1.0	0.0	26.7
Phomopsis sp.	7.0	0.9	0.7	1.3	0.0	26.7
Gliocladium sp.	1.3	0.7	0.0	1.7	0.0	20.0
Mycelia sterilia	1.0	1.0	1.3	0.3	0.0	26.7
Other fungi	3.3	2.2	1.0	2.3	0.0	–
Bacteria	10.7	7.0	2.0	9.0	0.0	80.0
Total	79.4	50.2	71.8	39.6	0.0	
Sterile pieces	32.3	54.0	35.3	67.7	100.0	
Total	111.7	104.2	107.1	107.3	100.0	

[1] Number of vines examined: 15.
[2] Based on 300 pieces of tissue for each decay type used for isolation.

Table 5. Identity and frequency of organisms accompanying *Stereum hirsutum* isolated from centrally light-colored necrosis of soft texture (Categories I and III),[1] zones a, b, c as shown in Figure 1A, h = healthy wood

Organisms isolated	Frequency (%)				
	a[2]	b[2]	c[2]	h[2]	T
Stereum hirsutum	70.0	8.7	2.3	0.0	100.0
Phaeoacremonium chlamydosporum	1.3	30.3	13.0	0.0	100.0
Phaeoacremonium aleophilum	0.0	24.7	17.3	0.0	100.0
Botryosphaeria obtusa	7.0	6.0	3.7	0.0	80.0
Gliocladium sp.	0.0	2.3	6.3	0.0	33.3
Alternaria sp.	2.3	1.3	0.7	0.0	33.3
Aspergillus sp.	1.7	1.7	3.0	0.0	26.7
Pestalotia sp.	1.7	0.3	1.0	0.0	20.0
Epicoccum sp.	2.7	0.0	0.3	0.0	4.0
Mycelia sterilia	3.7	2.7	2.7	0.0	53.3
Other fungi	5.0	2.3	6.3	0.0	–
Bacteria	1.7	2.0	11.0	0.0	100.0
Total	97.1	82.3	67.6	0.0	
Sterile pieces	12.3	23.0	33.0	100.0	
Total	109.4	105.3	100.6	100.0	

[1] Number of vines examined: 15.
[2] Based on 300 pieces of tissue for each decay type used for isolation.

Table 6. Identity and frequency of organisms accompanying *Stereum hirsutum* isolated from centrally discolored wood (Category III),[1] zones f, c as shown in Figure 1B, h = healthy wood

Organisms isolated	Frequency (%)			
	f[2]	c[2]	h[2]	T
Phaeoacremonium aleophilum	25.8	26.3	0.0	100.0
Phaeoacremonium chlamydosporum	22.5	17.5	0.0	100.0
Stereum hirsutum	15.0	1.2	0.0	100.0
Botryosphaeria obtusa	10.0	4.2	0.0	50.0
Gliocladium sp.	5.8	3.3	0.0	33.3
Aspergillus sp.	1.7	1.2	0.0	33.3
Other fungi	0.8	3.3	0.0	–
Bacteria	1.2	7.5	0.0	66.6
Total	82.8	64.5	0.0	
Sterile pieces	20.8	35.8	100.0	
Total	103.6	100.3	100.0	

[1] Number of vines examined: 6.
[2] Based on 120 pieces of tissue for each decay type used for isolation.

the time of inoculation. The wood formed after inoculation was entirely healthy. *S. hirsutum* induced a light-colored necrosis of soft texture and wood discoloration in young vines that was of the same color and intensity as that found in esca-affected vines. Necroses remained limited to the woody tissues present at the time of inoculation. Vines inoculated with *P. punctatus* showed brown-discolored stripes, 0.5–1 mm wide, in the xylem present at the time of infection. No stripes were observed in the wood formed after inoculation. This fungus could not be reisolated from the stripes.

None of the infected plants developed foliar symptoms typical of esca. All the young vines had a healthy vegetation.

Wood decay tests

Non-inoculated wood blocks were light brown and hard texture. Wood blocks inoculated with *P. aleophilum* were gray in color and soft in texture. *P. chlamydosporum* caused a green coloration and hard texture in wood blocks. *P. punctatus* and *S. hirsutum* produced a spongy, yellowish rot on wood blocks identical to the decay on standing vines.

Discussion

The examination of 309 diseased vines suggested the existence of two processes of wood colonization leading to tissue degradation.

The first process resulting in formation of central light-colored soft necrosis involves a number of microorganisms in sequence:

(1) *P. aleophilum* and *P. chlamydosporum* are encountered *in natura* in discolored wood in the center, and

(2) *P. punctatus* causes the typical decay associated with the disease, as indicated by the decay tests. Similar combinations of colonization leading to wood degradation have been described in many trees (Good and Nelson, 1962; Shigo, 1963). Invasion by pioneer fungi resulting in wood discoloration is apparently a necessary first stage before decay can develop. In this study, we cannot state that *P. punctatus* played a role in the first stage of wood degradation. Pathogenicity tests made with this fungus have failed. This is probably due to the fact that these plants did not constitute a good substrate for *P. punctatus*. Inoculations into older plants would be necessary to determine its possible pathogenic nature, and consequently its possible role in the first stage of the wood degradation.

The other fungus said to be responsible for esca, *S. hirsutum* (Vinet, 1909; Rives, 1921; Marsais, 1923; Viala, 1926), was not frequently isolated from the samples examined, only from 15 vines in 309 diseased plants. However, its role as a possible cause in the disease cannot be ignored. Pathogenicity tests showed it was involved in this disease, not only as a pioneer organism causing discolored necrosis in the center, but

Table 7. Symptoms caused by the principal fungi isolated from decayed wood of grapevine in artificial infection and reisolation. Number of plants inoculated: 30

Organisms	Characteristics of necroses	No[1]		Reisolation[2]	
		6 mo[2]	18 mo[2]	6 mo	18 mo
Phaeoacremonium aleophilum	Brown, soft, sectorial	16	8	+	+
Phaeoacremonium chlamydosporum	Brownish dark, dark, central	15	7	+	+
Phellinus punctatus	Brown discolored stripes	20	10	−	−
Stereum hirsutum	Brownish dark, hard, central light, soft, central	18	9	+	+
Control	Brownish dark near the point of inoculation	17	9	−	−

[1] Number of young plants which presented the necrosis described.
[2] + = reisolation, − = no reisolation.

also in the second stage of wood degradation. These findings are in agreement with those of Viala (1926) but differ from those of Chiarappa (1959) who considered it a saprophyte. Furthermore, as shown by microflora study, it could be associated with other microorganisms such as *P. aleophilum* and *P. chlamydosporum*.

The second process resulted in formation of sectorial light-colored soft necrosis from which *E. lata* and *P. punctatus* were isolated in high frequencies. *E. lata*, isolated from the wood surrounding the decay zone and from the sectorial brown necrosis, seems to play a role pioneer role in wood colonization. The parasitic nature of this fungus has been demonstrated by many studies on woody dicotyledonous hosts (Carter, 1957; Moller, 1964; English and Davis, 1965). It is also described as the causal agent of Eutypa dieback of grapevine (Moller and Kasimatis, 1978; Carter, 1991). *E. lata* can also pave the way of entrance to *P. punctatus*. Thus in the second process leading to wood degradation the sequence seems to be:

(1) colonization by *E. lata*, encountered *in natura* in sectorial necrosis, and
(2) *P. punctatus* causing the typical decay associated with the disease.

Since the beginning of the 20th century, esca was ascribed to *Phellinus igniarius* (L.: Fr.) Pat. (Ravaz, 1909; Baldacci et al., 1962; Chiarappa, 1959; Grasso, 1969) and *Stereum hirsutum* (Vinet, 1909; Rives, 1921; Marsais, 1923; Viala, 1926). The results reported here show that esca is in fact a more complex disease involving several microorganisms: *S. hirsutum, P. punctatus, P. aleophilum and P. chlamydosporum*. The first two fungi are responsible for wood degradation typical of esca. It is difficult to know if *P. punctatus*, which we have collected on standing vines, might have been mistaken for *P. igniarius* by others because little information was given on the morphological characteristics of basidiocarps by these authors. Resupinate forms of *P. igniarius* var. *viticidus* observed by Gard (1922), classified as *P. punctatus* (Larsen and Cobb-Poule, 1991), are similar to our basidiocarps observed on standing plants.

Two taxa of *Phaeoacremonium* are associated with these two basidiomycetes. *P. chlamydosporum* is similar to '*Cephalosporium* sp.' (Chiarappa, 1959) which has been considered to be close to *Phialophora parasitica* (Hawksworth et al., 1976). The presence of these two fungi in the discolored wood and the zones bordering the wood decay poses the question of the role of these two microorganisms in the disease complex. They must not be considered as secondary fungi, because they were able to colonize the living wood, as suggested by the pathogenicity tests. These results corrobate those of Chiarappa (1959) who showed that a '*Cephalosporium* sp.' was able to produce brown discolored wood.

Our study showed that *Eutypa lata* can also be associated with esca disease.

Despite the identification of all these microorganisms associated with esca in France, it is difficult to know if they are responsible for this disease, because the foliar symptoms have not yet been reproduced. Several *Phaeoacremonium* are known to cause withering of the various plants, identical to apoplexy in the case of esca. *Phaeoacremonium inflatipes* is responsible for withering of many species of oak (Halliwell, 1966). Thanassoulopoulos and Thanassoulopoulos (1984) add that *Phialophora parasitica*, which is considered as *Phaeoacremonium* sp. (Crous et al., 1996) is responsible for decline in olives and Rumbos (1986) reports this fungus as responsible for causing withering of *Prunus avium* L. Foliar symptoms could also be due to white rot fungi (*P. punctatus, S. hirsutum*). Further studies should be done in order to know the fungi

responsible for the foliar symptoms and the role of all these microorganisms in the disease. For that, inoculation tests with both types of colonizers in combination should be done.

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