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Differential aggressiveness of fungi implicated in esca and associated diseases of grapevine in France

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Summary. We tested differences in the aggressiveness of six fungal species, *Phaeomoniella chlamydospora, Phaeoacremonium aleophilum, Fomitiporia mediterranea, Eutypa lata, Diplodia seriata* and *Neofusicoccum parvum*, all of which are associated with esca, Eutypa dieback or 'black dead arm' (BDA) of grapevines in France. Ten isolates per species (nine isolates for *P. aleophilum*), originating in various regions of France, were tested on rooted cuttings of cv. Cabernet Sauvignon by inoculating the mycelium of the species on wounds made in the vine wood. The inoculated plants were incubated in an open greenhouse and the experiment was repeated twice. The isolates were divided into three groups depending on the severity of the infection. Infection severity was based on the extent of the external cankers and the internal lesions, measured 5 and 15 months after inoculation, as compared with the controls. The first group included all the *P. chlamydospora* and *N. parvum* isolates and two of the *E. lata* isolates. These isolates induced internal necrosis and external cankers developing from the point of inoculation. *P. chlamydospora* and *N. parvum,* produced large cankers and the longest internal lesions, and were the most virulent. The second group, comprising those *E. lata* isolates not in Group one and all isolates of *D. seriata* and of *P. aleophilum*, caused internal necrosis from which the vine afterwards totally healed. The third group included all the *F. mediterranea* isolates. These isolates caused the smallest lesions, generally not different from those in the controls, and developed a characteristic mycelium in the pith. No foliar symptoms were observed on any of the inoculated cuttings, except with the two *E. lata* isolates. Some seedlings displayed the typical foliar symptoms of Eutypa dieback. Different isolates within individual species exhibited significant differences ($P<0.05$) in lesion development. This made it possible to select the most aggressive isolates for further study.

Key words: *Vitis vinifera*, trunk disease, inoculation, rooted cutting.

Introduction

The fungal trunk diseases Eutypa dieback, esca and black dead arm, all of which occur in French vineyards, lead to dieback and the mortality of vines (Kobès *et al*., 2005). Several fungi cause, or are associated with, these diseases. Eutypa dieback is caused by the ascomycete *Eutypa lata*; this ascomycete develops in wood tissue and brings about sectorial brown necrosis. This wood necrosis is associated with symptoms such as smaller necrotic leaves and dwarfed shoots. Foliar symptoms are caused by toxic fungal metabolites such as eutypine and eutypinol and other related compounds transported via the vessels, or which interfere with nutrient transport by modifying vessel structure (Tey-Rulh *et al*., 1991; Molyneux *et al*., 2002; Mahoney *et al*., 2005; Lardner *et al*., 2006). The aetiology of esca of grapevine, which causes dieback in France and other European countries, is more complicated than that of Eutypa dieback. Esca has two types of ex-

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ternal symptoms, on the leaves (chronic and acute), caused by those fungi that are also associated with the various types of internal necrosis in vine wood. Esca is mainly associated with two ascomycetes, *Phaeomoniella chlamydospora* and *Phaeoacremonium aleophilum*, and one basidiomycete, *Fomitiporia mediterranea*. This last causes white rot (Larignon and Dubos, 1997; Mugnai *et al.*, 1999; Fischer and Kassemeyer, 2003; Fischer, 2006). Moreover, *E. lata* has also been isolated from brown necrotic lesions in vines displaying the external, visual symptoms of esca, further complicating the nature of the factors associated with this disease (Jamaux-Despréaux *et al.*, 1997; Larignon and Dubos, 1997; Péros *et al.*, 2008). *P. chlamydospora* and *Phaeoacremonium* spp. are also frequently isolated from young vines with decline or dieback: this condition has been termed Petri disease (Scheck *et al.*, 1998; Mugnai *et al.*, 1999; Edwards and Pascoe, 2004; Gimenez-Jaime *et al.*, 2006). The third type of dieback, black dead arm (BDA), has been reported from Hungary (Lehoczky, 1974) and France (Larignon *et al.*, 2001). With BDA, foliar symptoms are associated with a brown streak just under the bark, from which different Botryosphaeriaceae species were reported by different authors, such as *Botryosphaeria stevensii* (Lehoczky, 1974), *Botryosphaeria dothidea* and *B. obtusa* (Larignon *et al.*, 2001). The *B. dothidea* isolates collected by Larignon *et al.* (2001) in France were later identified as *Neofusiccocum parvum*. Recently, phylogenetic lineage analysis carried out on the Botryosphaeriaceae using DNA sequences of 28S rDNA (Crous *et al.*, 2006) has led to *B. obtusa* being named with the anamorph name *Diplodia seriata* only. Some species have been isolated from vines with BDA symptoms in Italy (Cristinzio, 1978), in Chili (Auger *et al.*, 2004), and from vines with esca symptoms (Larignon and Dubos, 1997; Armengol *et al.*, 2001; Fischer and Kassemeyer, 2003); therefore, discerning the number of species involved in esca disease remains a difficult task. Both Botryosphaeriaceae species, *D. seriata* and *N. parvum,* are also associated with various symptoms of dieback and with the deterioration of mature vines in most grapevine-producing areas around the world (Armengol *et al.*, 2001; Castillo-Pando *et al.*, 2001; Phillips, 2002; Auger *et al.*, 2004; Van Niekerk *et al.*, 2004; Taylor *et al.*, 2005; Van Niekerk *et al.*, 2006; Savocchia *et al.*, 2007; Urbez-Torres *et al.*, 2008).

The pathogenicity on grapevine of the fungal species associated with diseases causing grapevine dieback was tested using various plants and a number of inoculation methods. For instance the pathogenicity of *P. aleophilum* and *P. chlamydospora* was tested on grapevine callus tissue (Santos *et al.*, 2006), on seedlings in *vitro* (maintained alive) (Larignon and Dubos, 1997), on rooted cuttings of scions or rootstocks (Adalat *et al.*, 2000; Eskalen *et al.*, 2001), and on mature vines in a vineyard (Mugnai *et al.*, 1999; Adalat *et al.*, 2000; Sparapano *et al.*, 2001; Feliciano *et al.*, 2004). However, a few fungal species or only a few isolates of each species have been tested together in previous studies. The present study was undertaken to test a standardised method of inoculation, thus allowing an evaluation and comparison of the aggressiveness of the six species associated mainly with wood diseases, esca, BDA and Eutypa dieback in France. We also compared the behaviour of various isolates of each species in different areas of France.

Materials and methods

Fungal isolates and inoculum

We tested 59 isolates belonging to six fungal species collected from naturally infected grapevine trunks between 1988 and 2001: *Phaeomoniella chlamydospora*, *Phaeoacremonium aleophilum, Fomitiporia mediterranea*, *Eutypa lata*, *Diplodia seriata* and *Neofusicoccum parvum*. Ten isolates per species (nine isolates for Pal) were chosen from various geographical areas in France, except for one *E. lata* isolate, which originated in Italy (Table 1). All isolates were stored at 4°C in culture collections at INRA (Bordeaux, France) on malt agar (w: w, 3:4) slants. The fungal samples were incubated at 22°C (12h light/12h dark) on malt agar (w:w, 3:4) in Petri dishes before being used to inoculate various plants.

Seedling preparation and inoculation

In 2005 and 2006, a total of 720 cuttings cv. Cabernet Sauvignon collected from experimental vineyards at the INRA station in Couhins (Gironde, France) were calibrated according to their stem diameter (9 mm ±1 mm) and internode length (90 mm ±10 mm). They were disinfected in a solution of Cryptonol (3.5 l hl^{-1}, pH=7.5; Novartis Agro SA, Rueil-Malmaison, France) overnight. The cuttings

Table 1. Isolates of the six species included in this study.

Species	Isolate	Geographical origin (administrative district)	Sampling date, grape cultivar, former reference
P. chlamydospora	Pch 1	Moncaup (64)	1996, Cabernet Franc - AQ34 (Borie *et al.*, 2002)
P. chlamydospora	Pch 2	Julienne (16)	1996, Ugni Blanc
P. chlamydospora	Pch 3	Montbazillac (24)	1996, Sauvignon – AQ17 (Borie *et al.*, 2002)
P. chlamydospora	Pch 4	Ribeauvillé (68)	1996, Riesling
P. chlamydospora	Pch 5	Saint Laurent de C. (11)	1997, Grenache - LR81 (Borie *et al.*, 2002)
P. chlamydospora	Pch 6	Labastide d'Armagnac (40)	1996, Baco 22A - AQ31 (Borie *et al.*, 2002)
P. chlamydospora	Pch 7	Pouillac (17)	1996, Ugni Blanc – PC20 (Borie *et al.*, 2002)
P. chlamydospora	Pch 8	Rouffach (68)	1996, Gewurztraminer
P. chlamydospora	Pch 9	Martillac (33)	2004, Cabernet Sauvignon
P. chlamydospora	Pch 10	Espiet (33)	2004, Cabernet Sauvignon
P. aleophilum	Pal 1	Aydie (64)	1996, Tannat
P. aleophilum	Pal 2	Soublecause (65)	1996, Cabernet Franc
P. aleophilum	Pal 3	Julienne (16)	1996, Ugni Blanc
P. aleophilum	Pal 4	Les touches de Périgny (17)	1996, Ugni Blanc – PC1 (Borie *et al.*, 2002)
P. aleophilum	Pal 5	St Julien Beychevelle (33)	1996, Cabernet Sauvignon
P. aleophilum	Pal 6	Montbazillac (24)	1996, Sauvignon
P. aleophilum	Pal 7	Marlenheim (67)	1996, Riesling
P. aleophilum	Pal 8	Rouffach (68)	1996, Gewurztraminer
P. aleophilum	Pal 9	Assignan (34)	1997, Grenache - LR6 (Borie *et al.*, 2002)
F. mediterranea	Fm 1	St Christophe des Bardes (33)	1996, Cabernet Sauvignon
F. mediterranea	Fm 2	Jurançon (64)	1996, Gros Manseng
F. mediterranea	Fm 3	Pouillac (17)	1996, Ugni Blanc
F. mediterranea	Fm 4	St Christophe des Bardes (33)	1996, Cabernet Sauvignon
F. mediterranea	Fm 5	Zellenberg (68)	1996, Auxerrois
F. mediterranea	Fm 6	Eïchhoffen (67)	1996, Gewurztraminer
F. mediterranea	Fm 7	Leyrac (34)	1996, Cinsault, LR30 (Jamaux-Despréaux and Péros, 2003)
F. mediterranea	Fm 8	Macau (33)	2004, Cabernet Sauvignon
F. mediterranea	Fm 9	Moncaup (64)	1996, Cabernet Franc
F. mediterranea	Fm 10	St Prieul (16)	1996, Ugni Blanc
E. lata	El 1	Bordeaux (33)	1990, Cabernet Sauvignon, Bx 1-10 (Péros and Berger, 1994; Péros et al, 1999)
E. lata	El 2	St Christophe des Bardes (33)	1996, Cabernet Sauvignon
E. lata	El 3	Sud Est (France)	1991
E. lata	El 4	Verona (Italy)	1988
E. lata	El 5	Traenheim (67)	1996, Gewurztraminer
E. lata	El 6	Rouffach (68)	1996, Gewurztraminer
E. lata	El 7	Avisé (51)	2001
E. lata	El 8	Orbais (51)	-
E. lata	El 9	Julienne (16)	1996, Ugni Blanc
E. lata	El 10	Les touches de Perigny (17)	1996, Ugni Blanc

(continued on the next page)

(Table 1 continued)

D. seriata	Ds 1	Perpignan (66)	1998, Syrah - BoF98.1 (Larignon *et al.*, 2001)
D. seriata	Ds 2	Die (26)	1999, Clairette - BoF99.7 (Larignon *et al.*, 2001)
D. seriata	Ds 3	Naujean et Postiac (33)	1999, Cabernet Sauvignon
D. seriata	Ds 4	St Julien Beychevelle (33)	1999, Cabernet Sauvignon - BoF99.7 (Larignon *et al.*, 2001)
D. seriata	Ds 5	Avize (51)	2000, Pinot Meunier - BoF007 (Larignon *et al.*, 2001)
D. seriata	Ds 6	Ludon-médoc (33)	2000, Cabernet Sauvignon
D. seriata	Ds 7	Orbais (51)	2000 - BoF0014 (Larignon *et al.*, 2001)
D. seriata	Ds 8	Rodailhan (30)	1999, Syrah
D. seriata	Ds 9	Mardeuil (51)	2000
D. seriata	Ds 10	Sud Est (France)	1999, Syrah
N. parvum	Np 1	Nîmes (30)	2000, Syrah
N. parvum	Np 2	Armagnac (32)	2000
N. parvum	Np 3	Pauillac (33)	2000, Merlot
N. parvum	Np 4	Mardeuil (51)	2000
N. parvum	Np 5	Nîmes (30)	2000
N. parvum	Np 6	Blanquefort (33)	2000, Merlot
N. parvum	Np 7	St Julien Beychevelle (33)	1999, Merlot - Bd 1 (Larignon *et al.*, 2001)
N. parvum	Np 8	St Julien Beychevelle (33)	1999, Cabernet Sauvignon - Bd 3 (Larignon *et al.*, 2001)
N. parvum	Np 9	Epernay (51)	1999, Chardonnay – Bd 3.3 (Larignon *et al.*, 2001)
N. parvum	Np 10	Pauillac (33)	2000, Merlot

were rooted in a sandy layer at 25–30°C and irrigated daily with water for one month. They were then planted in 0.5 l plastic pots filled with Klassman RHP 15 commercial potting mix [fair peat of sphaine (70%), cold black peat (15%), pearlite and Danish clay (15%)], and were incubated for six weeks in a greenhouse (25°C, 16 h light/8 h dark). In 2005, a total of 490 rooted cuttings were inoculated with an isolate of the six tested species (10 cuttings per isolate and 10 isolates per species, except for *P. aleophilum* with 9 isolates, *D. seriata* with 5 isolates, and *N. parvum* with 5 isolates). In 2006, a total of 590 rooted cuttings were inoculated (10 cuttings per isolate and 10 isolates per species except for *P. aleophilum* with 9 isolates). The stem of each cutting was surface-sterilised with 95% ethanol and wounded with a 3-mm cork borer 4 cm below the upper bud. The hole of the wound was filled with a mycelium plug cut off from the margin of each fresh mycelial culture on malt agar, and the wound was sealed with paraffin wax. Ten rooted drilled cuttings were used as negative controls, into which non-colonised malt agar plugs were inserted. Ten rooted cuttings were not drilled, and served as controls to verify natural infection of the wood. The rooted cuttings were transplanted to 2.5 l plastic pots filled with Klassman RHP 15 potting mix two weeks after inoculation; the pots were incubated in an open greenhouse. Cuttings were randomly arranged within a species block and were fertilised with 2 g l^{-1} Peter's professional commercial growth mix once weekly. The samples were watered by sub-irrigation according to their requirements. The experiment was conducted twice: in the period between June 2005 and September 2006; and between May 2006 and October 2007. The daily temperatures were recorded throughout both tests using an electronic weather data logger.

Aggressiveness measurement and isolation

Discoloration or abnormality of the shoots and leaves was recorded during the spring and summer to search for Eutypa dieback, BDA, or esca foliar symptoms. The length and width of the external cankers of 5 plants were measured after bark removal five and 15 months after inoculation. The can-

ker surface areas were determined using the ellipse formula (πab, a=canker length/2, b=canker width/2). We analysed longitudinal and cross-sections of the cuttings at the point of inoculation, and recorded the colour, length and width of the lesions. Fungal samples were re-isolated using two previously inoculated cuttings, corresponding to each isolate; in the case of the controls, five cuttings were used for fungal re-isolation. Small pieces of wood isolated from the area of inoculation, from areas next to necrotic tissue and from non-discoloured tissue were separately disinfected in a solution of sodium hypochlorite (w:w, 2:100) for 45 s. These samples were then rinsed with sterile water and placed on malt-agar Petri dishes at 22°C (12 h light/12 h darkness per day), for the time necessary to identify the various characteristics of the species. For all species, the colony and conidial characteristics of the fungi isolated after the experiment were compared with those of the fungi used for inoculation.

Data analysis

Data were tested for normality using K. Pearson's skewness and kurtosis statistics. Analysis of variance (ANOVA) was performed to test for significant effects. Newman-Keuls tests were used to compare the means of significant effects and to determine the homogeneous grouping of the means using StatBox V6.6Pro (Grimmersoft [1997–2002] Paris, France). The non-parametric Mann Whitney test was used to compare data after 5 and 15 months, and the Wilcoxon test was carried out to compare the length of necroses above and below the point of inoculation. Non-parametric correlation, using the Spearman rank correlation coefficient was tested using R-programme 2.6.2 (http://www.r-project.org/).

Results

External and internal symptoms

External cankers were observed only after inoculation with the *N. parvum* and *P. chlamydospora* isolates and with two of the *E. lata* isolates (El1 & El3). Cankers appeared as early as five months after inoculation in the second trial (Fig. 1a). Five months after inoculation, the canker surface caused by *N. parvum* was larger than that caused by *P. chlamydospora*, with means of 80.7 mm^2 and 45.9 mm^2 respectively (Table 2). However, 72% of cuttings had cankers after inoculation with *N. parvum*, whereas 100% of cuttings had cankers after inoculation with *P. chlamydospora* (data not shown). The mean surface area of the cankers resulting from *N. parvum* inoculation increased from 80.7±11.06 mm^2 to 121.5±10.53 mm^2 between month 5 and month 15, whereas the size of *P. chlamydospora* cankers remained stationary. Some pycnidia were observed on cankers produced after inoculation with *N. parvum*. After bark removal, all inoculated cuttings without external cankers, as well as the drilled controls, showed total healing of the wounded tissue.

All inoculations caused internal necrotic lesions in the wood, differing in appearance and size depending on the species and the isolate. The longitudinal sections for most species had dark lesions proximal to the point of inoculation and long light-brown to black streaks extending upwards and downwards from the point of inoculation. Lesions remained localised at xylem tissue that had developed before inoculation (Fig. 1b). We observed a few individual black spots in sample cross-sections after inoculation with various isolates of *P. chlamydospora* (Fig. 1d). No lesions were observed in the undrilled controls. Longitudinal sections of drilled control cuttings revealed small lesions at the point of inoculation corresponding to the cork-borer wound.

Of the cuttings inoculated with *E. lata* isolates El1 and El3, only a few showed the typical foliar symptoms of Eutypa dieback one year later. Of the cuttings inoculated with the other species, none had the foliar symptoms typical of esca or BDA.

Comparison of internal necrosis length between species

Necrotic lesions in the drilled controls were limited, with a mean length of 3.3 mm and 3.8 mm, 5 and 15 months after inoculation respectively (Table 2). There were no significant differences ($P<0.05$) in the mean length of the necrotic lesions between the controls of the various species; this allowed a comparison of the internal lesions produced 5 and 15 months after inoculation in the second trial. Five months after inoculation, almost all fungal species had produced necroses reaching more or less the same distance upwards and downwards from the point of inoculation (data not shown); however, with *E. lata* isolates the basipetal lesions produced were longer than the acropetal lesions (Wilcoxon test, z=-2.76, $P=0.003$). Fifteen months after inoculation, the basipetal lesions were lon-

ger than the acropetal lesions with the *E. lata, F. mediterranea* or *D. seriata* isolates (Wilcoxon test, respectively z=-2.8; z=-2.65; z=-2.8; $P<0.004$), whereas with *N. parvum* isolates the basipetal lesions were shorter than the acropetal lesions (Wilcoxon test, z=2.19; $P=0.01$). For *P. chlamydospora* and *P. aleophilum*, there were no significant differences in the length of the necrosis above and below the inoculation site. After 5 months of incubation, *P. chlamydospora* and *N. parvum* produced the longest lesions, with identical means of 35.2 mm for both species (Table 2). Ten months later, the necroses produced by *P. chlamydospora* reached a mean length of 62.5 mm, whereas the necroses produced by *N. parvum* had a mean length of 45.1 mm. *D. seriata, E. lata* and *P. aleophilum* produced lesions with a length of between 12.3 mm and 14.5 mm after 5 months. These lengths had remained stationary after 5 and 15 months. *F. mediterranea* resulted in the smallest lesions, with a mean length of 4.4 mm and this changed only slightly between months 5 and 15. Cuttings inoculated with *F. mediterranea* had small black necrotic lesions in the xylem. Also, for 8 of the 10 isolates tested, the white mycelium that is characteristic of the fungus, and the lesion that looked like white rot, extended 46 mm above and below the point of inoculation in the pith (Fig. 1c).

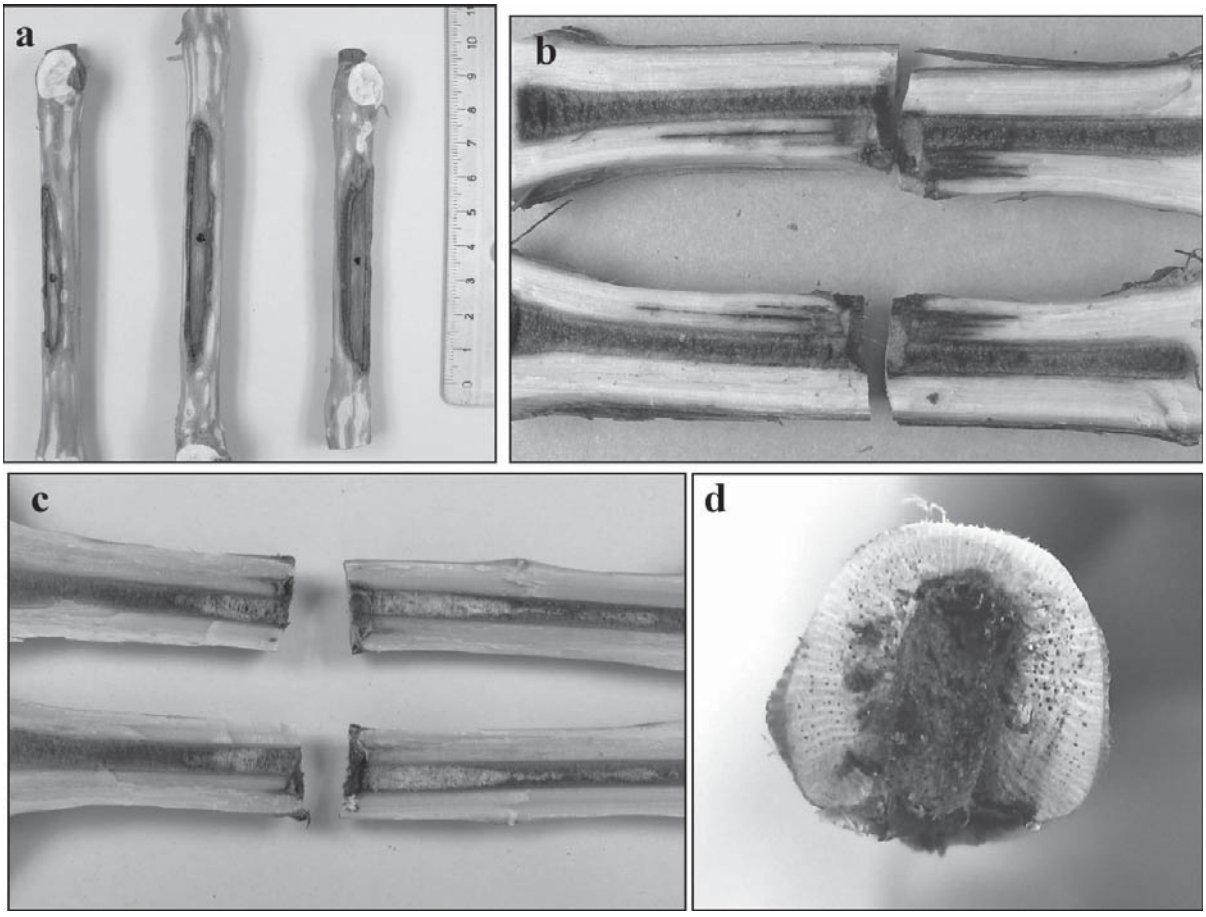

Fig. 1. Necrosis observed in wood of Cabernet Sauvignon cuttings. a, *Neofusicoccum parvum* cankers. b, Black streaks on longitudinal section following inoculation of *Neofusicoccum parvum*. c, Characteristic mycelium of *Fomitiporia mediterranea* into the pith on longitudinal section. d, Black spots on cross section following inoculation of *Phaeomoniella chlamydospora*.

Table 2. Surface of cankers and length of necrosis obtained at 5 and 15 months after inoculation (in 2006) of rooted cuttings cv. Cabernet Sauvignon with each of 6 fungal species.

Species	Mean[a] canker surface[b] (mm^2)		Mean[a] necrosis length (mm)	
	5 months	15 months	5 months	15 months
P. chlamydospora	45.9±1.80	41.4±1.30	35.2±1.80	62.5±1.67
P. aleophilum	No cankers		13.8±0.77	17.0±1.01
F. mediterranea	No cankers		4.4±0.20	6.2±0.34
E. lata	Small cankers [c]		14.5±0.79	13.6±0.76
D. seriata	No cankers		12.3±0.82	12.5±0.65
N. parvum	80.7±11.06	121.5±10.53	35.2±1.75	45.1±2.53
drilled Control (dC)	No cankers		3.3±0.21	3.8±0.38
not drilled Control (ndC)	No cankers		No necrosis	

[a] Data are means of five replicates per treatment ± standard deviation.
[b] Determined using the ellipse formula (πab, a=canker length/2, b=canker width/2).
[c] Cankers not measured, observed after inoculation of two isolates (El1 & El3).

Comparison of necrosis length within species

Five months after inoculation, all isolates of *P. chlamydospora* and almost all isolates of *N. parvum* (except isolate Np1) and *E. lata* (except isolate El4) produced significantly longer lesions ($P<0.01$) than those of the drilled controls (Fig. 2). The other species, *D. seriata*, *P. aleophilum* and *F. mediterranea* showed respectively 6, 7 and 8 isolates (of 10, or 9 in the case of *P. aleophilum*) that produced lesion lengths not significantly different from those of the drilled controls. After 15 months, inoculation with nine of the ten *F. mediterranea* isolates resulted in lesion lengths similar to those of drilled controls. Five isolates of *P. aleophilum* (Pal 3, 4, 6, 8, 9) and 2 isolates of *N. parvum* (Np 1, 2) also produced lesion lengths that were not significantly different from those of the drilled controls (Fig. 2). For the ten *P. chlamydospora* isolates, the lesion lengths were between 25.4 and 48 mm, and between 46.3 and 79 mm, 5 and 15 months after inoculation respectively (Fig. 2a). Statistical analysis of the isolates detected significant differences ($P<0.05$) in necrosis lengths both 5 and 15 months after inoculation. Samples inoculated with isolates Pch1 and Pch3 had the longest lesions. No significant correlation was found between the surface area of the cankers and the length of necrosis, with Spearman coefficients of -0.33 and -0.02, respectively, 5 and 15 months after inoculation. The various *N. parvum* isolates produced different lesion lengths (Fig. 2b), and these differences remained 5 and 15 months after inoculation. The lesion lengths were between 7 and 73 mm (5 months after inoculation), and between 14 and 73 mm (15 months after inoculation). Isolate Np10 produced the largest lesion, with a length equivalent to that between the two buds of the cutting. There was no significant increase in the length of the necrosis between 5 and 15 months after inoculation (Mann-Whitney test, z=-1.10, $P=0.14$). The internal necrosis was always longer than the corresponding canker. The surface area of the cankers resulting from *N. parvum* inoculation and the lengths of the corresponding internal lesions were significantly ($P<0.05$) correlated 5 and 15 months after inoculation, with Spearman coefficients of 0.72 and 0.75 respectively. In consequence of the great variability of necrosis length in *D. seriata* isolates, there was no significant isolate effect or date-of-inoculation effect on necrosis development, except with isolate Ds5. The nine isolates of *P. aleophilum* produced a mean necrosis length of between 11.2 and 18.4 mm 5 months after inoculation, and between 12.6 and 20.8 mm 15 months after inoculation (Fig. 2d). No significant differences were found between *P. aleophilum* isolates at any time during the study. The lesion length slightly increased between the two evaluation dates (Mann-Whitney test, z=-2.25, $P=0.01$). The lesion length produced by the *E. lata*

isolates was between 7.4 and 21.2 mm 5 months after inoculation, and were between 9.6 and 19.2 mm 15 months after inoculation (Fig. 2e). Five and 15 months after inoculation, isolate El1 produced the largest lesion, and isolate El7 induced a smaller lesion than the mean. The mean size of the lesions did not increase significantly between the first and second observation date (Mann-Whitney test, z=0.76, *P*=0.22). Lastly, all *F. mediterranea* isolates developed slight necrotic lesions in the xylem, with lengths between 3.4 and 6.2 mm, 5 months after inoculation. In the period following 15 months after inoculation, isolate Fm7 produced the largest lesion with a mean of 11.2 mm (Fig. 2f).

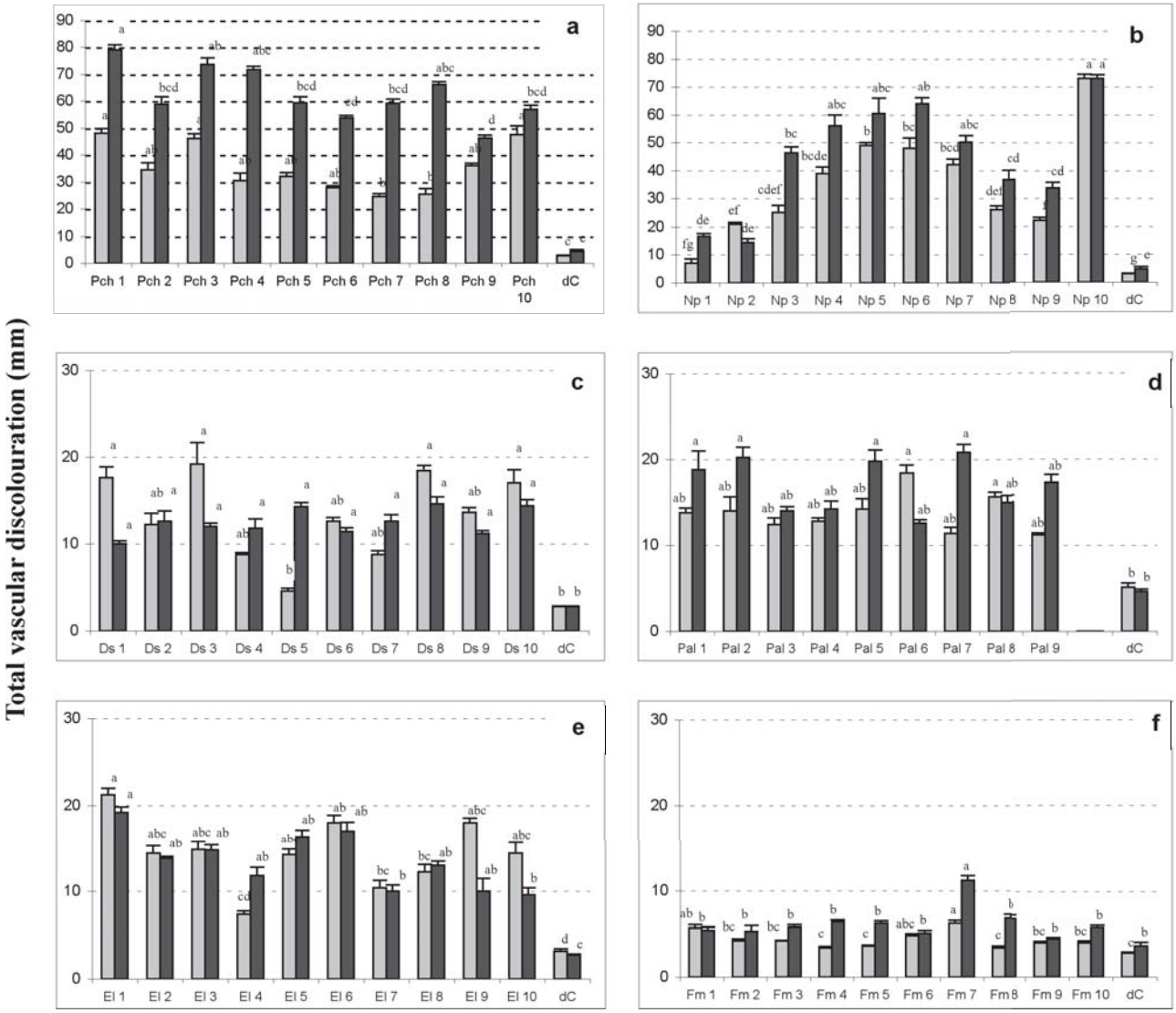

Fig. 2. Aggressiveness of the isolates of the six species tested in seedlings of cv. Cabernet Sauvignon compared to drilled control (dC): a, *Phaeomoniella chlamydospora* (Pch). b, *Neofusiccocum parvum* (Np). c, *Diplodia seriata* (Ds). d, *Phaeoacremonium aleophilum* (Pal). e, *Eutypa lata* (El). f, *Fomitiporia mediterranea* (Fm). Total vascular discolouration was the sum of the upward and downward lesions. Mean lesion length was based on five replicate isolates at each date (■ 5 months after inoculation - ■ 15 months after inoculation). Means followed by different letters are significantly (*P*<0.05) different according to Newman-Keuls test. Bars symbolise standard error of the mean.

Re-isolation of inoculated fungal species

The invasive capacity of each fungus was verified by re-isolating the inoculated species. All fungi inoculated were successfully re-isolated from every cutting at 5 months, with the exception of *F. mediterranea*, which was re-isolated from 16 of the 20 cuttings. *P. chlamydospora* and *D. seriata* were re-isolated respectively five and 15 months after inoculation from a single cutting (3%) of the undrilled controls (data not shown). Percent isolation of *P. chlamydospora* from the drilled controls was 27% at 5 months, and 13% at 15 months. Lastly, *D. seriata* was re-isolated from a single cutting of the drilled controls (data not shown). The rate of fungal re-isolation was dependent on the sampling site and the species used for re-inoculation (Table 3). Several other saprophytic fungi were also isolated at the point of inoculation: *Alternaria* sp., *Penicillium* sp., *Cladosporium* sp., *Chaetomium globosum, Aspergillus* sp., *Epicoccum* sp., and *Fusarium* sp., species that may be competitive with the fungi tested in this study. These saprophytic fungi were also isolated when the species tested were isolated from 100% of cases (*D. seriata* and *N. parvum*). *F. mediterranea* and *P. chlamydospora* were the species least frequently isolated at the point of inoculation. *P. aleophilum* and *P. chlamydospora* (≥90%) were most frequently re-isolated from surrounding necrotic areas and non-discoloured wood tissue; *F. mediterranea* had the lowest frequency of re-isolation, at about 20% (Table 3).

Comparison between trials

The behaviour of the six tested species was similar in both trials (Table 4). The temperature curves with monthly means during the two assays were generally similar in the 3 years, but in 2007 there was less variation in the temperature between winter and summer (Fig. 3). Within species, the classification of isolates varied with the dates of observation and with the trial (Table 4). The necrosis length of isolates after 15 months was generally more homogeneous than that after 5 months. However, this technique allowed us to identify one or two of the most aggressive isolates within each species. About *P. chlamydospora,* though there were no significant differences between isolates in the first trial, Pch1 persistently produced the largest necrotic lesion at three of the four inspection dates (Table 4). Necroses produced after inoculation with the nine *P. aleophilum* isolates in both trials differed significantly on only one observation date (15 months after inoculation, trial 1). Of the *P. aleophilum* isolates, Pal5 more often produced longer necrotic lesion than the other isolates. One isolate of *F. mediterranea* (Fm7) had the largest necrotic lesions, at three of the four inspection dates. Significant differences were found between the *E. lata* isolates, with El1 occurring in the largest necroses at three of the four inspection dates. Isolates El1 and El3 produced foliar symptoms in both experiments. We observed significant ($P<0.05$) differences among the five tested *D. seriata* isolates 5 months after inoculation in both trials. Ds3 produced the longest lesions, of great length, in the first trial (50.8 mm), whereas at 15 months, in the second, different trial, the mean lesion length was surprisingly only 6.4 mm. Ds5 consistently produced short lesions. We found significant differences in the necrotic lesion length after inoculation with five *N. parvum* isolates in the two trials; the lesion lengths in relation to the isolates varied on three of the four inspection dates. Isolate Np5 produced the longest lesion (Table 4).

Table 3. Percentage of reisolation of fungi for each species tested according to sampling site of isolation, 5 months after inoculation.

Species	Infected cuttings 5 months after inoculation (%)		
	At the point of inoculation (%)	At the margins of the wood discoloration (%)	In woody tissue not discoloured (%)
P. chlamydospora	15	95	90
P. aleophilum	83	89	94
F. mediterranea	20	22.5	20
E. lata	90	40	50
D. seriata	100	62.5	60
N. parvum	100	87.5	55

Table 4. Length of necrosis produced 5 or 15 months after inoculation of rooted cuttings cv. Cabernet Sauvignon with each isolate of the 6 species tested in the trials 1 (inoculation in 2005) and 2 (inoculation in 2006). Within a species, means followed by different letters differed significantly ($P<0.05$) according to the Newman-Keuls test. Means are expressed ± standard deviation.

Species	Isolate	Length of necrosis (mm)			
		5 months		15 months	
		Trial 1	Trial 2	Trial 1	Trial 2
P. chlamydospora	Pch 1	49.2 a ±4.1	48.0 a ±1.7	63.0 a ±4.0	79.0 a ±2.0
P. chlamydospora	Pch 2	42.6 a ±3.8	34.4 ab ±2.7	53.2 a ±2.2	58.8 bcd ±2.8
P. chlamydospora	Pch 3	45.0 a ±2.8	46.2 a ±1.6	59.6 a ±3.0	73.6 ab ±2.5
P. chlamydospora	Pch 4	40.6 a ±1.6	30.4 ab ±2.8	61.2 a ±3.4	71.8 abc ±1.1
P. chlamydospora	Pch 5	31.8 a ±1.5	32.0 ab ±1.3	48.6 a ±3.2	59.4 bcd ±2.2
P. chlamydospora	Pch 6	27.4 a ±2.6	27.8 ab ±0.8	54.8 a ±1.5	53.8 cd ±0.9
P. chlamydospora	Pch 7	21.0 ab ±2.6	24.6 b ±1.0	63.0 a ±2.1	59.2 bcd ±1.6
P. chlamydospora	Pch 8	31.4 a ±4.3	25.4 b ±2.0	69.0 a ±2.3	66.2 abc ±0.9
P. chlamydospora	Pch 9	40.0 a ±2.5	36.0 ab ±0.8	50.4 a ±2.4	46.3 d ±1.1
P. chlamydospora	Pch 10	36.8 a ±1.4	47.6 a ±3.2	54.8 a ±1.7	57.0 bcd ±1.6
drilled Control		2.8 b ±0.1	2.8 c ±0.1	2.6 b ±0.1	3.0 e ±0.6
Mean		36.58	35.24	57.66	62.51
P. aleophilum	Pal 1	10.2 ab ±0.1	13.8 ab ±0.5	13.0 ab ±0.9	18.8 a ±2.2
P. aleophilum	Pal 2	6.8 ab ±1.0	14.0 ab ±1.6	10.2 b ±0.8	20.2 a ±1.2
P. aleophilum	Pal 3	7.0 ab ±0.2	12.4 ab ±0.8	13.2 ab ±0.7	14.0 ab ±0.5
P. aleophilum	Pal 4	7.1 ab ±0.4	12.8 ab ±0.4	8.2 b ±0.5	14.2 ab ±0.9
P. aleophilum	Pal 5	24.6 a ±2.7	14.2 ab ±1.2	21.8 a ±1.4	19.8 a ±1.3
P. aleophilum	Pal 6	11.6 ab ±1.6	18.4 a ±1.0	13.8 ab ±0.9	12.6 ab ±0.3
P. aleophilum	Pal 7	20.2 ab ±2.6	11.4 ab ±0.7	15.4 ab ±2.0	20.8 a ±0.9
P. aleophilum	Pal 8	24.8 a ±3.7	15.6 a ±0.6	12.2 ab ±1.0	15.0 ab ±0.8
P. aleophilum	Pal 9	8.6 ab ±0.6	11.2 ab ±0.2	12.2 ab ±0.3	17.3 ab ±0.9
drilled Control		2.0 b ±0.0	5.1 b ±0.5	4.0 b ±0.3	4.6 b ±0.3
Mean		13.43	13.76	13.33	16.96
F. mediterranea	Fm 1	3.6 bc ±0.4	5.7 ab ±0.4	6.6 ab ±0.3	5.4 b ±0.4
F. mediterranea	Fm 2	3.8 bc ±0.4	4.2 bc ±0.2	4.3 ab ±0.3	5.3 b ±0.7
F. mediterranea	Fm 3	6.4 ab ±0.2	4.2 bc ±0.1	6.4 ab ±0.3	5.8 b ±0.2
F. mediterranea	Fm 4	3.5 bc ±0.3	3.4 c ±0.2	7.6 ab ±0.9	6.4 b ±0.2
F. mediterranea	Fm 5	6.6 ab ±0.2	3.6 c ±0.1	4.4 ab ±0.3	6.2 b ±0.3
F. mediterranea	Fm 6	5.6 bc ±0.3	4.8 abc ±0.2	6.6 ab ±0.2	5.1 b ±0.3
F. mediterranea	Fm 7	8.8 a ±0.7	6.2 a ±0.3	8.8 a ±0.4	11.2 a ±0.5
F. mediterranea	Fm 8	4.6 bc ±0.2	3.4 c ±0.2	8.6 a ±0.6	6.8 b ±0.5
F. mediterranea	Fm 9	3.3 bc ±0.2	4.0 bc ±0.1	7.4 ab ±0.3	4.4 b ±0.2
F. mediterranea	Fm 10	5.6 bc ±0.3	4.0 bc ±0.2	9.6 a ±1.1	5.8 b ±0.2
drilled Control		2.6 c ±0.2	2.8 c ±0.1	2.4 b ±0.1	3.6 b ±0.4
Mean		5.18	4.35	7.17	6.24

(continued on the next page)

(Table 4 continued)

E. lata	El 1	14.6 a ±0.6	21.2 a ±0.8	17.6 ab ±1.0	19.2 a ±0.6
E. lata	El 2	10.4 ab ±0.7	14.4 abc ±0.9	22.2 a ±0.9	13.8 ab ±0.2
E. lata	El 3	10.2 ab ±0.6	14.8 abc ±0,9	14.8 abc ±0.8	14.8 ab ±0.6
E. lata	El 4	6.7 b ±0.8	7.4 cd ±0.4	8.8 c ±0.4	11.8 ab ±1.0
E. lata	El 5	6.4 b ±0.3	14.2 abc ±0.7	15.0 abc ±0.8	16.4 ab ±0.7
E. lata	El 6	7.6 b ±0.9	18.0 ab ±0.8	15.0 abc ±1.1	17.0 ab ±1.1
E. lata	El 7	5.4 b ±0.2	10.4 bc ±0.9	18.6 ab ±0.6	10.0 b ±0.7
E. lata	El 8	6.3 b ±0.3	12.2 bc ±0.9	11.2 bc ±0.5	13.0 ab ±0.4
E. lata	El 9	8.2 b ±0.3	18.0 abc ±0.5	13.4 bc ±0.5	10.0 ab ±1.4
E. lata	El 10	9.0 b ±0.6	14.4 abc ±1.2	14.4 bc ±0.7	9.6 b ±0.8
drilled Control		1.0 c ±0.1	3.2 d ±0.2	3.6 d ±0.2	2.8 c ±0.1
Mean		8.48	14.50	15.10	13.56
D. seriata	Ds 1	6.2 b ±0.7	17.6 a ±1.2	9.8 a ±0.8	10.0 a ±0.3
D. seriata	Ds 2	17.2 b ±1.4	12.3 ab ±1.3	16.2 a ±1.6	12.6 a ±1.2
D. seriata	Ds 3	50.8 a ±5.7	19.2 a ±2.4	6.4 a ±0.4	12.0 a ±0.4
D. seriata	Ds 4	20.4 b ±3.5	8.8 ab ±0.2	20.4 a ±3.5	11.8 a ±1.0
D. seriata	Ds 5	4.6 b ±0.4	4.6 b ±0.3	4.6 a ±0.4	14.3 a ±0.5
drilled Control		4.8 b ±0.4	2.8 b ±0.8	3.2 a ±0.2	2.8 b ±0.7
Mean		19.84	12.33	11.48	12.49
N. parvum	Np 1	29.0 ab ±2.2	7.0 d ±1.5	34.2 b ±3.5	16.6 b ±1.0
N. parvum	Np 2	35.4 a ±5.3	21.0 cd ±0.6	17.6 bc ±1.5	14.4 b ±1.4
N. parvum	Np 3	11.0 ab ±1.0	25.0 bc ±2.5	11.2 bc ±1.0	46.3 a ±2.2
N. parvum	Np 4	37.6 a ±4.3	39.0 b ±2.3	28.6 bc ±2.6	56.0 a ±3.9
N. parvum	Np 5	25.5 ab ±1.6	49.0 a ±1.0	58.4 a ±4.4	60.4 a ±5.6
drilled Control		4.8 b ±0.4	3.0 e ±1.8	3.2 c ±0.2	4.8 b ±2.5
Mean		27.70	35.24	30.00	45.16

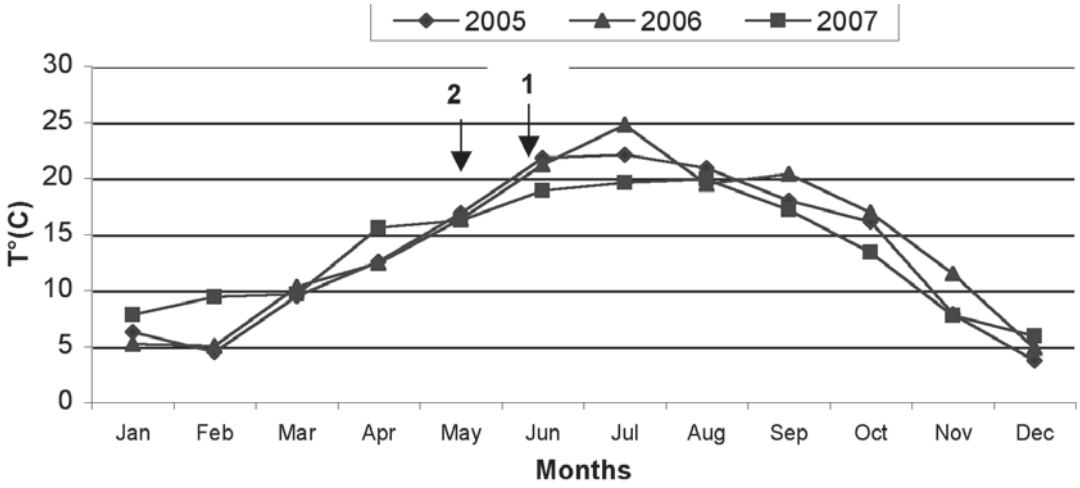

Fig. 3. Monthly temperature curves in 2005, 2006 and 2007. Arrows 1 and 2: date of inoculation in 2005 and 2006 respectively.

Discussion

This is the first study to compare the aggressiveness of six major fungal species associated with, or causing grapevine wood diseases in France, in controlled conditions with artificial inoculation of rooted Cabernet Sauvignon cuttings. We distinguished several groups of fungal isolates based on observable symptoms. All *P. chlamydospora*, *N. parvum* isolates and some *E. lata* isolates producing internal necrotic lesions and cankers were the most virulent isolates. A second group comprised all the *D. seriata* and *P. aleophilum* isolates and the other *E. lata* isolates; these also produced internal necrosis, but this was followed by the total healing of the wounds. Lastly, *F. mediterranea* isolates, which induced less extensive necroses, generally not different from those of controls and which grew in the pith, were weak pathogens or not pathogenic within the conditions outlined in this study. These findings were confirmed by an analysis of ten isolates per species (except for *P. aleophilum*, for which 9 isolates were tested), and observations carried out at different times.

The comparison between the two inspection dates (5 and 15 months after inoculation) allowed an evaluation of the speed of fungal colonisation in the wood, and plant reaction. Thus, all *N. parvum* isolates caused severe symptoms with large cankers as early as 5 months after inoculation. These symptoms changed slightly between the two inspection dates. However, the extent of symptoms varied depending on the isolate. *N. parvum* was more aggressive than the other Botryospheaeriacea, *D. seriata*, judging by how early the symptoms appeared, and how severe they were. This finding was consistent with those obtained by Phillips (2002), who reported more severe symptoms, such as cane bleaching and wood streaking, with *N. parvum* than with *D. seriata* in grapevines.

We observed cankers after inoculation with *P. chlamydospora*. This fungus occurs on the bark and in pruning wounds, and has been isolated in its latent or endophytic form from internal necrotic tissue and from apparently healthy wood (Larignon and Dubos, 1997; Scheck *et al.*, 1998; Ferreira *et al.*,1999; Mugnai *et al.*, 1999), but it has not been isolated from the external cankers. However, in contrast to *N. parvum*, the amount of internal necrosis was not correlated with the external cankers. These severe symptoms may have been related to the experimental conditions used in this study, such as the use of a susceptible cultivar Cabernet Sauvignon, and/or the high inoculum pressure brought to bear on the wound tissue. When comparing *P. aleophilum* and *P. chlamydospora*, which are both thought to be the identified causes of esca, pathogenicity tests showed that all *P. aleophilum* isolates caused necrotic lesions with a shorter mean length than did *P. chlamydospora* isolates, and did not induce cankers. Other studies of comparative pathogenicity, using one isolate of each species, also showed that *P. chlamydospora* produced longer lesions (Adalat *et al.*, 2000; Halleen *et al.*, 2007). The nine isolates of *P. aleophilum* tested in this study, which came from various regions of France, confirmed that under the experimental conditions used, *P. aleophilum* was less pathogenic than *P. chlamydospora*.

The aggressiveness of *E. lata* is generally evaluated according to the percentage of plants on which it causes foliar symptoms (Péros and Berger, 1994, Péros *et al.*, 1997; Péros *et al.*, 1999; Sosnowski *et al.*, 2007). However, in our study only two isolates of *E. lata* induced foliar symptoms, although they did cause internal brown necrosis. The failure of *E. lata* to express foliar symptoms after its artificial inoculation on young plants has been previously reported by several authors (Peros and Berger, 1994; Peros *et al.*, 1997; Sosnowski *et al.*, 2007). This non-expression of foliar symptoms may be related to a loss of toxin production by the isolates, due to their lengthy period of storage. Others factors related to experimental conditions may also explain this weakness in foliar symptom expression. Péros and Berger (1994) used unrooted cuttings directly placed in a water-saturated inert substrate, which may have been more favourable to the establishment and growth and toxin migration of *E. lata* than the conditions in our study. To compare the aggressiveness of *E. lata* isolates, we measured the length of the necrotic lesions produced: these showed significant differences between isolates. The isolates producing the longest lesions also induced cankers at the inoculation site, and caused foliar symptoms to be expressed in a few plants. Indeed, isolates El1 & El3 may be considered the most pathogenic *E. lata* isolates. Isolate El1 corresponds to the isolate used in previous studies (Peros and Berger, 1994) where it was also found to be one of the most aggressive isolates.

The fact that all re-isolated fungi used for

inoculum came from the edge of discoloured wood showed that these necroses were caused by living fungi. All tested fungi were also isolated from non-discoloured tissue at similar rates to those isolated from the edge of discoloured wood. This finding shows that the fungi spread out in living tissue without causing visible cell damage, and provided a clearer indication of the time period between the spread of the fungus and the reaction of the plant. The rates of re-isolation were relatively high for all fungi, except *F. mediterranea*. In comparison with the other fungi, the small lesions produced after the inoculation of this fungus may have contributed to its low re-isolation. This may be explained by the inability of this fungus to invade the plant tissues as an initial infectious agent. Larignon and Dubos (1997) suggested that *F. mediterranea* behaved as a secondary fungus, degrading the host cell walls only after the living tissue had first been invaded by *P. aleophilum*, *P. chlamydospora* or *E. lata*. *F. mediterranea* is mostly considered to be a saprophyte or a hemisaprophyte (Boulet, 2003). However, in grapevine, Sparapano *et al*. (2000) considered that *Fomitiporia punctacta* (former name for *F. mediterranea*) acted as a primary invader. The poor growth of this fungus in rooted cuttings may be related to the ontogenic resistance of young tissues in the host plant. Sparapano *et al*. (2000) showed that *F. punctata* colonised adult grapevines more rapidly when it was inoculated into the vine trunk than when it was inoculated into the spurs.

We observed the symptoms in the wood (cankers and internal necrosis) as early as five months after inoculation, and thus it was possible to discriminate the most aggressive species and isolates. The second experiment confirmed the first as regards species aggressiveness, and enabled one or two of the most aggressive isolates to be identified. Foliar symptoms were not seen in any test or at any inspection date, except with two *E. lata* isolates. Other factors, not controlled in our study, may have to concur to cause the typical foliar symptoms. Dark necrosis was observed in xylem tissue when longitudinal sections of the stems were examined. This allowed us to evaluate the ability of invasion as a component of the aggressiveness of the fungi. Discoloration, corresponding to phenolic compounds produced by the plant in response to infection, appeared to be a non-specific response to any aggressor or wound. The tolerance of the plant was related to its capacity to build defence barriers against the invasive fungi. This standardised method of inoculation may be sufficiently robust to test the grapevine response and, for instance, to test the various grapevine genotypes. However, Sosnowski *et al*. (2007) reported that it is necessary to take account of the age of the vine, as this affects the response to infection. In conclusion, the standardised method of inoculation presented here is repeatable and is sufficiently robust to characterise the degree of fungal pathogenicity based on the symptoms seen in the wood. We found a range of aggressiveness between and within species with the two most pathogenic fungi, *P. chlamydospora* and *N. parvum*, that could be used for future studies.

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