WOOD NECROSIS IN ESCA-AFFECTED VINES : TYPES, RELATIONSHIPS AND POSSIBLE LINKS WITH FOLIAR SYMPTOM EXPRESSION

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Abstract

Aims : Esca disease of grapevine is characterised by foliar symptoms associated with the development of various internal wood necroses. The aims of the present study are to determine the type and the quantity of necroses in the various woody compartments of vines, the relationships between them and the links between necroses and severity of foliar symptoms.

Methods and results : Symptomatic and asymptomatic vines cv Cabernet-Sauvignon were cross-sectioned to quantify the different types of internal necrosis in the scions (cordons, heads, and trunks) and rootstocks. Five necrosis « variables » were accounted for: central necrosis, sectorial necrosis, mixed necrosis, white rot, altered perimeter and in addition to the variable healing cone. In the scion, for all types of necrosis variables, a significant correlation between compartments was found. Vines with acute foliar form of esca had very advanced peripheral tissue degradations in the xylem and cambial zones. Chronic foliar expression of esca was associated with quantity of internal necroses higher than those obtained for asymptomatic vines. A logistic model indicated that white rot in the cordons was the best predictor for the chronic form of esca.

Conclusion : Necroses formed a continuum within the plant. The scion is like a single unit with a volume of necroses useful to determine the health status of vines.

Significance and impact of the study : A quantitative analysis of vine internal necroses would open up new possibilities for esca-epidemic approaches.

Key words : grapevine, esca disease, foliar symptoms, internal woody necroses, multivariate statistical analyses

Résumé

Objectifs : La maladie de l’esca de la vigne est caractérisée par des symptômes foliaires associés au développement de différentes nécroses internes du bois. Les objectifs de cette étude sont de déterminer le taux et les types de nécroses dans les différents compartiments ligneux de la vigne, les relations entre eux et les liens existant entre les nécroses du bois et la sévérité des symptômes foliaires.

Matériel et résultats : Des vignes, de cv. Cabernet-Sauvignon, symptomatiques et asymptomatiques sont sectionnées transversalement pour quantifier les différents types de nécrose du greffon (cordon, tête de souche, tronc) et du porte-greffe. Cinq variables « nécrose » sont définies : nécrose centrale, nécrose sectorielle, nécrose mixte, pourriture blanche, périmètre altéré, en plus de la variable cône de cicatrisation. Dans le greffon, il est montré une corrélation significative entre compartiments pour toutes les variables « nécrose ». Les vignes exprimant la forme sévère de l’esca présentent une forte dégradation des tissus ligneux périphériques comprenant la zone cambiale et la partie externe du xylème. La forme chronique de l’esca est associée à plusieurs types de variables « nécrose » de taille significativement plus importante que celles observées dans les vignes asymptomatiques. L’application d’un modèle logistique indique que la pourriture blanche est le meilleur prédicteur pour la forme chronique de l’esca.

Conclusion : Les nécroses du bois forment un continuum à l’intérieur du greffon, celui-ci pouvant être considéré comme une unité caractérisée par un certain volume de nécrose, utile pour déterminer l’état sanitaire de la plante.

Signification et impact de l'étude : Cette analyse quantitative des nécroses internes de la vigne pourrait ouvrir sur de nouvelles possibilités d’approches épidémiologiques de l’esca.

Mots clefs : vigne, maladie de l’esca, symptôme foliaire, nécroses internes du bois, analyse statistique multivariée.

AP, altered perimeter ; C, healing cone ; CN, central necrosis ; MN, mixed necrosis ;
NDT, non-discoloured tissue ; P, black punctate necrosis ;
SN, sectorial necrosis ; WR, white rot

manuscript received 12th August 2011 - revised manuscript received 27th February 2012

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INTRODUCTION

Esca syndrome induces a decline in vine vigour that generally ends up with the death of the plants. It has a complex aetiology, involving various biotic factors (pathogenic fungi), and possibly abiotic factors associated with cultivation (Mugnai *et al*., 1999; Graniti *et al*., 2000). This syndrome is of particular concern because it is widespread in the different wine producing regions of European countries, e.g., France (Fussler *et al*., 2008; Larignon and Dubos; 1997), Spain (Gimenez-Jaime *et al*., 2006), Greece (Rumbos and Rumbou, 2001), Italy (Surico *et al*., 2000), Portugal (Mendes *et al*., 2003), Germany and Switzerland (Fischer and Kassemeyer, 2003), Austria (Reisenzein *et al*., 2000) and Hungaria (Rabai *et al*., 2008). Esca and the closely related Petri disease have also been reported in many other vine-growing regions of the world (Edwards and Pascoe, 2004 ; Halleen *et al*., 2003; ; Ridgway *et al*., 2002; Scheck *et al*., 1998). A lack of knowledge concerning the origin and the epidemiology of this disease has hampered attempts to develop efficient control methods. Diagnosis is complex with the disease displaying diverse external symptoms. Two main forms of the disease have been described : (i) a chronic (or slow) form, involving a characteristic discolouration of the leaves and (ii) an acute (or apoplectic) form, characterised by a sudden drying out of the leaves during the hot season, rapidly leading to the death of the vine (Mugnai *et al*., 1999).

These foliar symptoms are associated with the development of internal wood necroses caused by various pathogenic fungi. *Phellinus igniarius* and *Stereum hirsutum* were the first basidiomycetes isolated from soft rot, also sometimes referred to as « amadou » (Ravaz 1898; Viala, 1926). Since the early nineties, several other fungi have been reported to be involved in the development of various types of necrosis, including *Phaeomoniella chlamydospora*, *Phaeoacremonium aleophilum* and *Fomitiporia mediterranea*. *P. chlamydospora* has been often isolated from dark streak necroses and central pink-coloured necrosis, and also from other necroses. *P. aleophilum* is one of the dominant species isolated from central pink-coloured or brown necrosis, along with *P. chlamydospora*, and from sectorial necrosis and black spots (Luque *et al*., 2009). *F. mediterranea*, often misidentified as the closely related species *F. punctata* (former *Phellinus punctatus*) (Fischer, 2000) is mainly isolated from white rot in European vineyards (Armengol *et al*., 2001; Cortesi *et al*., 2000; Fischer, 2006; Larignon, 1991; Larignon and Dubos, 1997; Mugnai *et al*., 1999). *Eutypa lata* is isolated from sectorial brown necrotic area, and different species of Botryosphaeriaceae, e.g., *Diplodia seriata*, are associated to brown to grey-brown necrotic area and brown stripes in trunks expressing symptoms of esca (Fischer and Kassemeyer, 2003 ; Kuntzmann *et al*., 2010 ; Luque *et al*., 2009, Romanazzi *et al*., 2009 ; Wunderlich *et al*., 2011). The frequency of isolation of each fungus from the different types of necrosis varies according to variety, geographical location and agricultural practices.

The causes of foliar symptoms remain an important area of research. Potentially pathogenic fungi produce several metabolites with phytotoxic properties (Evidente *et al*., 2000; Luini *et al*., 2010; Sparapano *et al*., 2000a; Tabacchi *et al*., 2000). Some of them produced by *P. chlamydospora* and *P. aleophilum* have been recovered from the xylem sap of esca-infected vines (Bruno and Sparapano, 2006, 2007) or detected in symptomatic leaves (Andolfi *et al*., 2009). It was suggested that the phytotoxic compounds produced by lignicolous fungi were transported to non-woody tissues via the xylem and caused the foliar necrosis, because no fungi were recovered from the leaves.

Esca is thus a complex disease and the dynamics of necrotic development in the woody tissues remains largely speculative (Larignon and Dubos, 1997; Surico *et al*., 2005). Furthermore, the manifestation of the foliar symptoms may fluctuate from year to year. This led Marchi *et al*. (2006) to coin the term « hidden esca » to describe the disease observed one year but not the year after. In that context, it is difficult to estimate the real incidence of the disease from the annual expression of symptoms on the leaves. It is therefore more appropriate to consider the cumulative incidence of esca over a period of at least three to five years (Surico *et al*., 2000) and to calculate prevalence.

While it is difficult to assess the real incidence of the disease by observing the foliar symptoms, the actual severity of the disease, taking into account the amount of necroses in the wood, is even more difficult to determine because it is hidden. Alternatively, a detailed quantification of internal necroses in the various parts of the vine would provide a real measurement of the disease severity. It is also more informative to determine the quantitative relationships between the internal wood necroses due to pathogenic fungi and the severity of symptoms on leaves. Such a quantitative approach would open up new possibilities for epidemiologic approaches to esca disease, involving modelling and prediction. To date, several studies have attempted to establish a relationship between the extent and type of necrosis in the wood and the foliar symptoms, but often with contradictory results. In a review on esca, Mugnai *et al*. (1999) described various types of necrosis and suggested that the level of expression of foliar symptoms only partly reflected the extent of necrosis. In another study, Calzarano and Di Marco (2007) evaluated the severity of foliar symptoms as a percentage of the total area of the crown of vines and they estimated visually the percentage of affected area. They found no significant correlations between the severity of foliar symptom expression and the severity of internal necroses in the two Italian vine cultivars studied (cv. Trebbiano d'Abruzzo and cv. Sangiovese). Péros *et al.* (2008) found no relationship between the severity of foliar symptoms and specific lesions or detection of pathogenic fungi. However, in a recent publication, Luque *et al.* (2009) noticed that internal symptoms were the result of multiple diseases coexisting in the same vine and showed that the type of internal symptom did not correspond to the external ones in only 10 % of the vines. Overall, these studies point out the complex nature of these symptoms and a clarification about this puzzling issue is needed.

In the present paper, a detailed analysis of the necroses formed in Cabernet-Sauvignon vines (from vineyards in the Bordeaux region) with and without the expression of foliar symptoms has been carried out. The objectives were (i) to determine the type and the quantity of necroses in the various compartments of the vines (cordon, head, trunk and rootstock) and to study the relationships between them, (ii) to quantify the various forms of internal necroses by comparing vines displaying different levels of severity of esca foliar symptoms: the acute form, the chronic form (with different multi-annual frequency expression) and the asymptomatic ones, and (iii) to identify the most accurate indicators of the variable « internal necrosis » accounting for foliar symptoms. To achieve these goals several multivariate statistical methods, including logistic regression analyses, were tested using two samples of vines taken from vineyards of the Bordeaux region in France.

MATERIALS AND METHODS

1. Vine samples and severity of esca foliar symptoms

Two kinds of vine samples were used for the experiments. The first one, called the « multiplot sample », was made up of vines taken from 20 vineyards in six areas of the Bordeaux region (South West of France, Table 1). The multiplot sample was used to quantify the internal necroses of the various compartments of the vines. The second sample, called the single-plot sample, was collected from a single vineyard in the Bordeaux region (Ludon-Médoc, Gironde) and was used to quantify the internal necroses in young and adult vines and to study the multi-annual frequency expression of chronic esca foliar symptoms.

The 20 vineyards used for the multiplot sample were planted with Cabernet-Sauvignon (*Vitis vinifera* L), which is susceptible to esca disease. The vines were grafted on various rootstocks (101-14 Mgt, Kober 5BB, Paulsen 1103, 3309 Couderc, Riparia Gloire and SO4) between 1987 and 1990 and trained according to the Guyot method. About 2000 vines from each vineyard were surveyed for esca disease at two different periods in 2004 : May/June and at the end of August. The plants had either acute or chronic forms of esca (foliar symptoms) or were

Table 1 - Characteristics of the 20 vineyards in the Bordeaux region (France) monitored and used for the multiplot sampling of vines.

Vineyard	Region	Commune	Year of Planting	Rootstock	Incidence of esca in year 1			Number ofvines collected		
					% chronic	% acute	% total	Asymptomatic	Esca chronic form	Esca acute form
1	Médoc	Margaux	1988	3309 Couderc	5.54	1.23	6.77	2	4	1
2	Médoc	Margaux	1987	101-14 Mgt	2.05	0.05	2.10	4	4	0
3	Médoc	Margaux	1987	101-14 Mgt	2.99	0.05	3.04	4	2	0
4	Médoc	Macau	1990	3309 Couderc	11.15	1.70	12.85	16	11	7
5	Pessac-Léognan	Canéjan	1988	-	2.95	0.45	3.40	0	1	0
6	Pessac-Léognan	Léognan	1988	101-14 Mgt	0.00	0.10	0.10	0	0	1
7	Pessac-Léognan	Martillac	1988	Riparia Gloire	0.84	0.64	1.48	0	0	3
8	Graves	Castres	1989	3309 Couderc	10.65	0.76	11.41	4	4	2
9	Graves	Beautiran	1989	-	2.96	0.39	3.35	5	2	1
10	Entre-deux-mers	Espiet	1986	SO4	4.40	0.54	4.94	1	1	1
11	Entre-deux-mers	Espiet	1986	SO4	3.90	0.20	4.10	1	1	1
12	Saint-Émilion	Saint-Émilion	1989	101-14 Mgt	0.84	0.25	1.09	3	7	1
13	Saint-Émilion	Saint-Émilion	1989	101-14 Mgt	0.05	0.25	0.30	3	5	1
14	Libournais	Saint-Philippe d'Aiguille	1987	101-14 Mgt	0.07	0.34	0.41	0	0	1
15	Libournais	Capitourlan	1989	101-14 Mgt	0.16	0.08	0.23	0	0	1
16	Libournais	Guîtres	1990	SO4	32.40	3.30	35.70	0	0	7
17	Libournais	Guîtres	1989	SO4	37.00	4.99	41.99	0	0	7
18	Libournais	Guîtres	1987	SO4	28.10	0.60	28.70	0	0	1
19	Libournais	Galgon	1987	Kober 5BB	3.30	0.75	4.05	4	7	2
20	Libournais	Galgon	1987	3309 Couderc and Paulsen 1103	11.07	1.00	12.07	3	6	0
Mean or total					8.02	0.88	8.90	50	55	38

asymptomatic. The incidence of esca disease in these vineyards was between 0.1 and 42 %.

A multiplot sample of 143 vines (either symptomatic or asymptomatic) was collected in February 2005: 55 vines displaying the chronic foliar form of esca on at least one cordon, 38 vines displaying the acute foliar form on both cordons, which is considered as the most severe form of esca, and 50 vines displaying no foliar symptoms. Eighty-seven (34 with chronic form, 24 with acute form and 29 asymptomatic vines) of these 143 vines were excavated with their rootstocks and the remaining 56 sectioned just above the graft union. One to 32 vines per vineyard were collected, depending on the number of vines with acute form of esca and whether permission could be obtained from the vintner to collect asymptomatic vine samples and vines with rootstock.

The single-plot samples were collected from a vineyard planted in 1981 with Cabernet-Sauvignon grafted onto rootstock 101-14 Mgt and trained according to the Guyot method. From 2003 to 2005, 517 vines were analysed for foliar symptoms and mortality (following esca expression) at the end of August. The esca severity was expressed in terms of multi-annual frequency of chronic disease expression on the leaves.

On the basis of their symptoms, 137 scions were collected in October 2005 by sectioning the vine just above the graft union. Six categories were defined: 32 vines with chronic form of esca recorded at least once (for 14 vines, esca was recorded in only one year, E1; for 14 vines, esca was recorded in two years, E2; and for 4 vines, esca was recorded in each of the three years, E3); 24 vines with one or two dead cordons (with esca disease noted during the study period), DA; 27 young vines (one or two years old) without esca foliar symptoms (Y); and

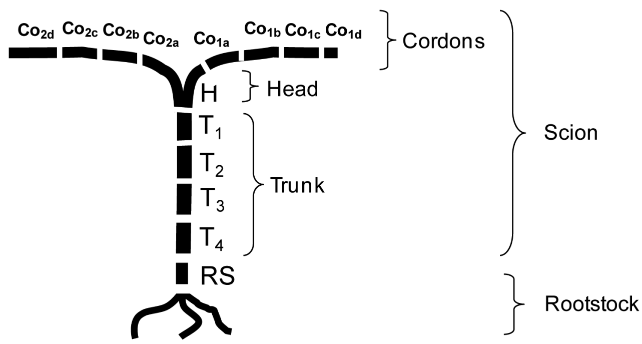

Figure 1 - Diagram of vine compartments: cross-sections were cut from rootstock (RS), head (H), trunk (T) and both cordons (Co), at 6-cm intervals.

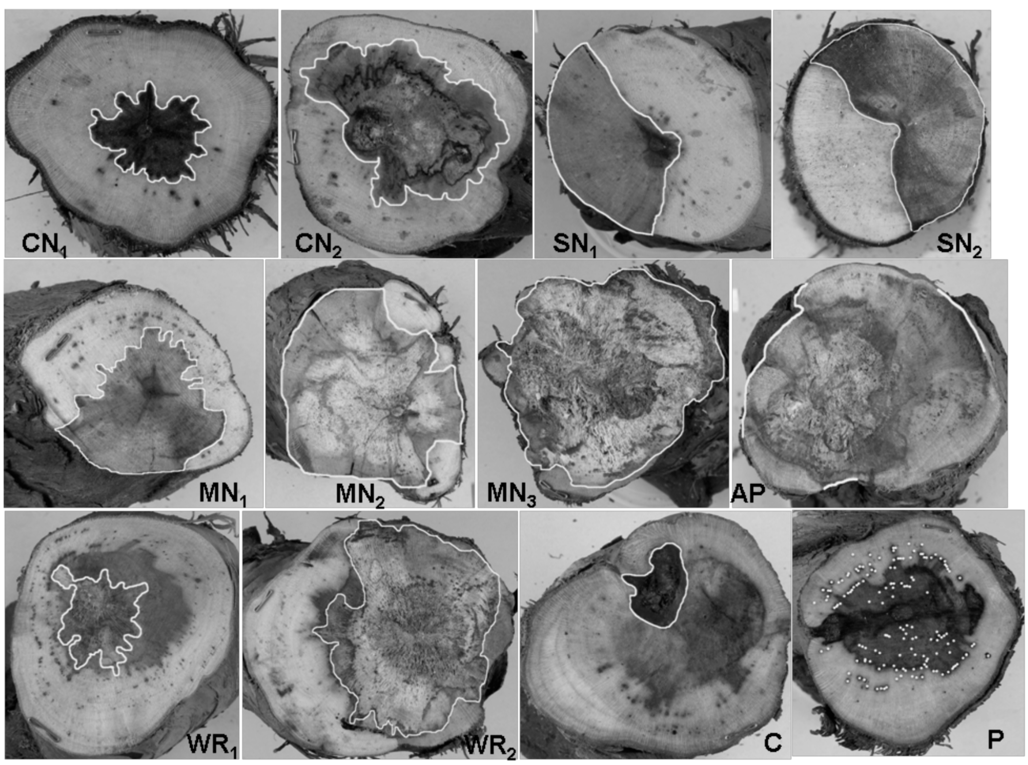

Figure 2 - Digital photographs of cross-sections, analysed with image processing software (Optimas 6.51) to quantify the relative area (% of total area) of each type of necrosis defined by Larignon and Dubos (1997).

The coloured line delimits the area of the measured variables: CN1, CN2: central necrosis; SN1, SN2: sectorial necrosis; MN1, MN2, MN3: mixed necrosis; WR1, WR2: white rot; C: healing cone. The length of the variable altered perimeter (AP) was measured and the number of black punctate necroses (P) was counted.

54 adult vines that remained asymptomatic during the three years studied (NS).

2. Quantification of internal necrosis

Within 24 hours of sampling, the vine samples were washed, dried, and cross-sections of the rootstocks, trunks, cordons and head zone were cut at 6-cm intervals with a circular saw (Figure 1). Each cross-section was immediately photographed with a digital camera (Nikon Coolpix 5700). Optimas image analysis software (version 6.51, Media Cybernetics) was then used to measure the area covered by each type of necrosis on each image, with a total of 3465 images analysed. Five types of necrosis were considered, as defined by Larignon and Dubos (1997) (Figure 2) : (i) black punctate necrosis (P), the spots were counted and the total number of spots was divided by the total area of necrosis to give a relative extent of necrosis ; (ii) central necrosis (CN), with no development of necrosis at the periphery of the wood section ; (iii) sectorial necrosis (SN), in which the margin of the necrosis followed the transverse vessels of the wood ; (iv) mixed necrosis (MN), corresponding to a combination of CN and SN when these two types were too difficult to separate ; and (v) white rot (WR), characterised by yellow or white soft rot and a healing cone (C). The relative area covered by these necroses was calculated by dividing the necrotic area by the total area of the wood section. The altered perimeter (AP), corresponding to the external perimeter of SN or MN, was also measured. The relative length was calculated by dividing AP by the total perimeter of the wood section.

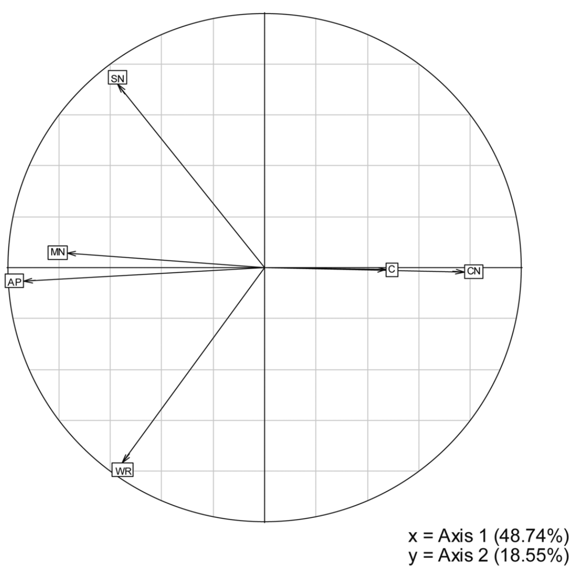

Figure 3 - Projection of wood necrosis variables onto the factorial plane for the first two factors of the principal component analysis

(CN = central necrosis ; SN = sectorial necrosis ; MN = mixed necrosis, AP = altered perimeter, WR = white rot ; C = healing cone).

3. Fungal isolation from necroses

Just after photographing, each type of necrosis was analysed for the presence of fungal pathogens in symptomatic and asymptomatic vines from the multiplot sample. After the removal of a thin wood layer, the cross-section was superficially disinfected with 70 % ethyl-alcohol, then small pieces of necrotic tissue (cut at the lesion margin) and non-discoloured tissue were separately disinfected in a solution of sodium hypochlorite (w : w, 2 : 100) for 45 secondes and rinsed with sterile water. These pieces were cut into about 5 mm^3, plated onto malt-agar in Petri dishes kept at about 24 °C and examined daily for the presence of fungal colonies. After sub culturing the emerging colonies on malt agar, the colonies were identified based on morphological and cultural features.

4. Data analysis

Statistical analyses were performed with R 2.8.1 software (R Development Core Team 2008). Non-parametric tests were used because the data were not normally distributed. The differences in internal necrosis between the different vine compartments (rootstocks, trunks, cordons, and head zone) were assessed by Friedman's ANOVA, with $P < 0.01$ considered as significant. The necrotic areas measured from scions of different vine categories were compared by Kruskal-Wallis ANOVA followed by a multiple comparison test, with $P < 0.01$ considered as significant. The relationship between necroses from different vine compartments was analysed by carrying out Spearman correlation analyses for particular variables. Correlations were considered significant with $P < 0.01$.

Principal component analysis was used to study the relationship between the internal wood necroses and the foliar symptoms. The data set included 3 categories of variables identifying foliar symptoms, *i. e.*, acute form of esca, chronic form of esca and non-symptomatic. Six categories were used to characterise the internal necroses, *i. e.*, CN, SN, MN, AP, WR and C. Quantitative variables corresponded to relative areas of necrosis or relative length of AP. The resulting data matrix contained 143 individual vines and 6 variables. The principal components were obtained by computing the eigenvalues and eigenvectors of the data correlation matrix. A bi-dimensional representation of this multidimensional set was made for the principal components that accumulated a significant percentage of original information.

Logistic regression was used to identify the internal necrosis variables significantly associated with the

J. Int. Sci. Vigne Vin, 2012, **46**, n°1, 15-27

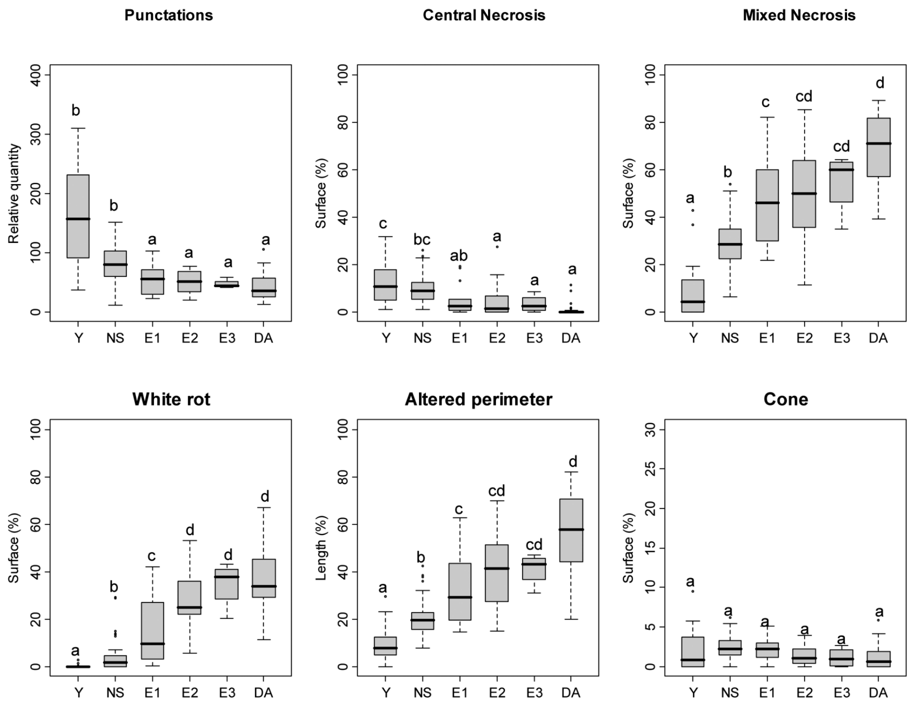

Figure 4 - Box plots representing quantitative values of each mesured variable (punctations, central necrosis, mixed necrosis, white rot, altered perimeter, and healing cone) to compare six categories of vines: asymptomatic young vines (Y), asymptomatic adult vines (NS), vines expressing chronic esca symptoms on the leaves in one year (E1), vines expressing chronic esca symptoms on the leaves in two years (E2), vines expressing chronic esca symptoms on the leaves in three years (E3), and vines with one or two dead cordons (DA). Box plots with different letters are significantly different according to the Kruskal-Wallis Test ($P < 0.01$).

Table 2 - Percentage of the total cross-sectional area of wood corresponding to each type of necrosis, in each compartment of the vine. Values are means (+/- standard deviation) and medians of 87 vines with rootstock from the multiplot sample

(CN = central necrosis, SN = sectorial necrosis, MN = mixed necrosis, WR = white rot, AP = altered perimeter, C = healing cone). Means with different letters are significantly different (Kruskal-Wallis test $P < 0.001$).

Necrosis variable		Vine compartment			
		Rootstock	Trunk	Head zone	Cordons
CN	Mean % ± SD	5.8 ± 6.9 a	4.9 ± 4.8 a	6.4 ± 8.6 a	6.0 ± 5.3 a
	Median %	3,2	3.7	2.8	4.7
SN	Mean % ± SD	0.4 ± 3.1 a	4.3 ± 12.7 bc	5.0 ± 17.4 ab	5.6 ± 12.2 c
	Median %	0	0	0	0
MN	Mean % ± SD	2.6 ± 6.9 a	8.0 ± 12.7 b	12.3 ± 19.4 b	9.4 ± 16.1 b
	Median %	0	1,8	0	2.0
WR	Mean % ± SD	2.5 ± 8.0 a	5.0 ± 9.3 b	9.6 ± 18.5 b	6.29 ± 11.3 b
	Median %	0	0	0	0
Total necrosed surface	Mean % ± SD	8,8 ± 9,3 a	17.3 ± 18.5 b	23.8 ± 22.9 c	21.0 ± 18.8 c
	Median %	6.2	12.7	16.5	14.2
AP	Mean % ± SD	9.9 ± 13.5 a	17.8 ± 21.6 ab	26.2 ± 31.2 b	35.3 ± 27.1 c
	Median %	4.7	8,9	12,4	25.0
C	Mean % ± SD	5.0 ± 6.8 b	3.6 ± 2.8 b	3.6 ± 7.2 a	13.4 ± 6.9 c
	Median %	2.3	2.8	0	12.9

expression of foliar symptoms (Mila *et al*., 2004; Fussler *et al*., 2008). The dependent variable was the absence or presence of esca symptoms on the vine. Colinearity between the independent candidate variables was first explored by Pearson's correlation analysis (a threshold for R coefficient < 0.7). Multivariate logistic regression was carried out with explicative or independent variables (necrosis variables) for which a P value less than 0.25 was obtained in preliminary univariate logistic regressions (Hoswer and Lemeshow, 1989). Multivariate logistic models were tested with external symptom type (chronic esca, apoplectic esca) used as a binary response or dependent variable (presence or absence of the symptom concerned), for both the single-plot and multiplot data (Mila *et al*., 2004 ; Fussler *et al*., 2008). Independent variables were considered to be significantly associated with the dependent variable (foliar symptom) when P < 0.01. A backward elimination method was used to select the best predictors. Akaike's information criterion (AIC) was used to compare the fits of the different models. Models were ranked in decreasing order of AIC value, corresponding to increasing goodness of fit (Eyre, 2007). Odds ratios (OR) were calculated to compare the strength of retained associations. The independent variable acts as a significant risk factor if OR > 1 and that the lower bound of the confidence interval does not go below 1.

RESULTS

1. Multiplot sample analysis : Distribution of necroses in rootstocks and scions

The subset of 87 vines from the multiplot sample (143 total vines) had internal necroses. The necroses were variable in extent and their types varied between compartments (Table 2). P, not quantified for this sample, was observed very frequently on cross-sections, either dispersed or evenly clustered along a short line (Figure 2). For each variable, the mean was higher than the median, indicating that the data were not normally distributed. In particular, the null values of the medians revealed that, for at least 50 % of the vines, SN, MN and WR were not present in one of the compartments.

In the rootstock, CN occupied a larger area (5.8 %) than the other types of necrosis (0.4-2.6 %), and the total necrotic area (8.8 %) was significantly smaller (P < 0.01) than in the other compartments (17.3-23.8 %). In the scion, necrosis affected between 0.02 and 85.88 % of the cross-sections, with a mean value of 19.3 % (data not shown). The total necrotic area increased from the bottom to the top of the vine, with values of AP and C significantly greater in the cordons (P < 0.01) than in the other compartments.

A significant correlation (P < 0.01) between the various compartments was found for all variables except for C. Spearman's correlation coefficient was between 0.28 and 0.63, depending on the variable and the compartments that were compared (Table 3). The strongest relationship was found for CN, between trunk and rootstock, and for AP, between cordons and trunk. A significant correlation (P < 0.01) was shown between the two cordons for all variables other than C.

2. Multiplot sample analysis : Internal necroses in vines with chronic or acute esca and in asymptomatic vines

Based on the mean data per scion for each internal necrosis variable, PCA revealed that the two highest-ranking components accounted for 67.29 % of the variance, with the first component accounting for 48.74 % of the variance (Figure 3). On the first axis, CN and C, with positive co-ordinates, were opposed to the variables MN, AP, SN and WR, with negative co-ordinates. Three variables, CN, MN and AP, contributed strongly to axis 1, with a positive correlation between MN and AP. The variables WR and SN contributed principally to axis 2, and were opposed on this axis. The variable C was strongly associated with axis 3 (data not shown).

Table 3 - Relationship between mean relative cross-sectional area or length in one compartment (rootstock, trunk, cordons, cordon 1 or cordon 2) and that in another compartment for the six variables : central necrosis (CN), sectorial necrosis (SN), mixed necrosis (MN), white rot (WR), altered perimeter (AP) and healing cone (C), from the multiplot sample. Values are Spearman's correlation coefficients (* P < 0.01).

	Rootstock *vs*. Trunk	Trunk *vs*. Cordons	Cordon 1 *vs*. Cordon 2
Central necrosis	0.57*	0.41*	0.40*
Sectorial necrosis	0.38*	0.45*	0.34*
Mixed necrosis	0.41*	0.43*	0.40*
White rot	0.45*	0.43*	0.40*
Altered perimeter	0.28*	0.63*	0.54*
Cone	-0.01	0.05	0.19

The group of « acute esca » (apoplectic) vines had the largest relative area of MN and the longest relative length of AP with significant values ($P < 0.001$) (Table 4). The group of « chronic esca » vines had intermediate values of CN and WR, lying between those of the other two foliar groups, but were not significantly different from the non-symptomatic vine group with respect to SN, MN, AP and C.

3. Multiplot sample analysis: Fungal isolation from necroses

The frequency of the isolated fungi varied from 0 to 82.7 %, depending on the necrotic type and the vine type (Table 5). *P. chlamydospora* was mainly isolated from P but was found in all types of necrosis, even in non-discoloured tissue (NDT), whereas *P. aleophilum*, *F. mediterranea* and *E. lata* were mainly isolated from CN, WR and SN, respectively. The frequency of Botryosphaeriaceae was particularly great in SN (26.5-41.5 %), and reached 23.8 % in NDT of vines with acute esca form. Other fungi like *Alternaria* spp., *Aspergillus* spp., *Epicoccum* spp., *Fusarium* spp., *Penicillium* spp., and *Trichoderma* spp. were also isolated. The frequency of these saprophytes was high in necroses sampled from vines with acute esca form.

4. Single-plot sample analysis: necroses in young and adult vines

Among the 517 vines observed from the single vineyard, 22.3 %, 31.3 % and 22.4 % expressed foliar symptoms in 2003, 2004 and 2005 respectively (data not shown). The six categories of vines defined in terms of age and external symptoms showed different amounts of necrosis (Figure 4). Scions of young vines, none of which displayed any foliar symptoms, were characterised by high relative levels of P. For adult vines, the median values of the variables MN, AP and WR increased with the severity of disease expression on the leaves. For these three variables, asymptomatic vines were significantly different ($P < 0.01$) from both vines displaying chronic symptoms (E1, E2, E3) and vines with dead cordons (following disease expression). The considerable dispersion of the values of these variables was obtained around the median. The area of WR was significantly ($P < 0.01$) smaller in E1 than in E2 and E3 vines. No significant difference was found for the variable C. The area of CN, considered alone, was smaller in symptomatic adult vines (E1, E2, E3, and DA) than in asymptomatic vines (Y, NS), with a significant difference ($P < 0.01$).

5. Modelling of the relationship between external and internal symptoms

Based on the five variables selected (CN, MN, WR, AP and C), the results from the multiple logistic regression indicated that acute foliar form of esca could be predicted by one variable, AP, with a significant P value (Table 6). The most discriminative value of AP for separating vines with and without symptoms was determined as 30 %: 78 % of acute-esca vines and 20 % of asymptomatic vines had an AP of at least 30 % ($\chi^2 = 21.17$, $P < 0.001$).

For chronic esca, three (WR, P and CN) of the four variables considered (P, CN, WR and C) were retained, but a significant P value was obtained only for WR. The amount of WR in the graft increased the risk of expression of the slow form of esca. A threshold value of 10 % necrosis can be used to distinguish vines with chronic esca symptoms from vines without chronic esca. The frequency of soft rot was greater than 10 % in 75 % of vines with esca symptoms, while 87.7 % of the plants without symptoms had a soft rot frequency below 10 %

Table 4 - Mean and median values of each measured variable (CN = central necrosis, SN = sectorial necrosis, MN = mixed necrosis, AP = altered perimeter, WR = white rot, C = healing cone) in the scion for the three categories of vines in the multiplot sample, defined in terms of foliar symptoms.

Foliar symptom†	Internal necrosis variables					
	CN	SN	MN	WR	AP	C
Asymptomatic	$5.93^{a‡§}$	2.91^{a}	7.42^{b}	4.20^{b}	19.39^{b}	8.01^{a}
(N=50)	5.42¶	0	5.64	0.70	14.05	7.16
Chronic form	4.64^{ab}	4.52^{a}	7.36^{b}	6.45^{ab}	21.29^{b}	7.77^{a}
(N=55)	4.55	0	6.21	3.69	16.95	8.31
Acute form	2.98^{b}	6.90^{a}	18.85^{a}	14.77^{a}	44.80^{a}	6.71^{a}
(N=38)	1.80	0.97	15.68	7.44	41.96	6.27

†N = Number of vines; ‡Mean value; ¶Median value
§Means with different letters are significantly different (Kruskal-Wallis test $P < 0.001$)

(χ^2 = 77.39, P < 0.001). « WR » was also the best descriptor when the analysis was carried out taking necrosis variables into account for the « cordons » component.

DISCUSSION

1. Continuum of wood necrosis in the vines

The analysis of wood sections from various compartments of Cabernet-Sauvignon vines (rootstocks, trunks and cordons, and head zone) indicated that several types of necrosis developed frequently in both esca-symptomatic and asymptomatic vines. Necroses generally corresponded to the descriptions made by Larignon and Dubos (1997). A pathogenic fungus predominated in each type of necrosis (Larignon and Dubos, 1997 ; Kuntzmann *et al*., 2010 ; Péros *et al*., 2008), however, it was pointed out that necroses were colonised by a complex of several fungal species, either potentially pathogenic or saprophytes or even potentially plant protectors, e.g., *Trichoderma* spp. The relationships between these fungi in the wood of vines still remain to be investigated (Bruez *et al*., 2011).

Necroses were present in each of the vine compartments analysed, including the rootstock, enabling for the first time to study their relationships between compartments. CN predominated in the rootstock and seemed to develop from the trunk down to the rootstock. In these vines, which were trained according to the Guyot method, there was generally more necrotic tissue in the cordons than in the other compartments. This was certainly due to pruning, which generated wounds facilitating the entry of pathogenic or saprophytic fungi, and allowed multiple infections over the years. In line with this assumption, it has been reported in the literature that pruning wounds are susceptible to infection by *P. chlamydospora* and *P. aleophilum* (Gubler *et al*., 2001 ; Larignon and Dubos 2000). The amount of necrosis in the cordons and in the trunk was correlated, suggesting that the various wood necroses formed a continuum within the plant. The scion may thus be considered as a single unit characterised by a volume of necroses useful for determining the health status of the vine.

2. Wood necroses and the acute and chronic forms of esca

Wood necroses were generally more extensive in vines displaying the acute form of esca than in vines displaying the chronic form, particularly in the cordons. In addition, wood necroses were less developed in adult and young asymptomatic plants. The acute esca form was frequently associated with an advanced stage of degradation of peripheral tissues : the AP. This wood zone is made of functionally important tissues, i. e., the newly formed xylem and cambium. Necrosis development in AP may result in the transpiration requirements of the aerial parts of the plant not being met due to the lack of water transport via the xylem. Consistent with this notion, apoplectic forms are frequently observed in the vineyards

Table 5 - Frequency of fungi isolated from necrotic and asymptomatic tissues of vines taken from the multiplot sample and displaying or not esca foliar symptoms.

Foliar symptom†	Wood¶ necrosis	Number of isolations	Isolated fungi					
			Phaeomoniella chlamydospora	*Phaeoacremonium aleophilum*	*Fomitiporia mediterranea*	*Eutypa lata*	Botryosphaeriaceae	Others
Chronic form (N=46)	P	159	69.2	5.0	0.0	1.3	4.4	5.0
	CN	38	39.5	31.6	2.6	5.3	18.4	36.8
	SN	82	13.4	6.1	6.1	17.1	41.5	18.3
	WR	84	30.9	16.7	58.3	3.6	8.3	8.3
	NDT	15	13.3	0.0	0.0	0.0	6.7	13.3
Acute form (N=27)	P	109	64.2	6.4	0.0	0.9	7.5	12.8
	CN	18	22.2	27.8	0.0	11.1	33.3	38.9
	SN	83	6.0	19.3	4.8	15.7	32.5	26.5
	WR	40	10.0	5.0	62.5	7.5	5.0	20.0
	NDT	21	23.4	4.8	0.0	0.0	23.8	14.3
Asymptomatic (N=20)	P	75	82.7	6.7	0.0	0.0	0.0	6.7
	CN	32	50.0	40.6	3.1	6.3	3.1	9.4
	SN	68	10.3	25.0	0.0	22.1	26.5	27.9
	WR	9	22.2	11.1	77.8	0.0	22.2	11.1
	NDT	16	18.8	0.0	0.0	0.0	0.0	25.0

†N = Number of vines used for isolation ; ¶Type of wood necrosis : P (black punctate necrosis), CN (central necrosis), SN (sectorial necrosis), WR (white rot), NDT (non-discoloured tissue)

Table 6 - Parameter estimates (with SEM and significance) for the best-fit binary regression logistic model predicting the probability of occurrence of esca expression from wood necrosis variables for single-plot or multiplot sample. Significant values of P : *P < 0.01, **P <0.001, ***P < 0.0001.

Data source	Dependent variable	Independent variables considered	Independent variables retained	Estimated coefficient ± SE	P value	Odds ratio	Odds Ratio 95% confidence interval
Multiplot scion	Logit(P(Apo) > 0)	CN + MN + WR + AP + C	(Intercept)	-2.49 ± 1.04	0.017		
			AP	0.09 ± 0.01	0.003*	1.07	1.04-1.11
Single-plot scion	Logit(P(Esca) > 0)	P + CN + WR + C	(Intercept)	0.20 ± 1.26	0.873		
			P	-0.02 ± 0.02	0.199	0.98	0.95-1.00
			WR	0.11 ± 0.03	0.001**	1.12	1.05-1.21
Single-plot Ind. cordon	Logit(P(Esca) > 0)	P + CN + WR + AP + C	(Intercept)	-2.07 ± 1.01	0.040		
			WR	0.11 ± 0.02	3.21e-09***	1.13	1.09-1.17

during summer, after heavy rain, when transpiration demand is high (Surico *et al*., 2005). Here, the leaf response, characterised by the total and rapid drying of leaves, may be compared with the symptoms of folletage, a physiological disorder in which the balance between water uptake and evapotranspiration is disturbed (Viala, 1926 ; Galet 1977). However, Letousey *et al*. (2010) recently demonstrated that two water stress-related genes (TIP1 & PIP2.2) were not affected in pre-apoplectic grapevine leaves (drying leaves), suggesting that the pre-symptomatic signals may be more strongly linked to pathogenic fungi than to water stress.

Concerning the chronic form, the chronic esca-vines sampled from the single vineyard had more severe internal necroses than asymptomatic vines. However, no significant differences were obtained between the vines with the chronic form of esca and the asymptomatic vines from the multiplot data. It is suggested that the sampling of vines from vineyards with differences in disease incidence may generate a higher level of variability, erasing the differences. Results from our study differ from those obtained by Calzarano and Di Marco (2007), who found no relation between the severity of wood deterioration and the severity of external foliar symptom expressed as a percentage of the crown area. Furthermore, they frequently detected black streak necroses in cordons. In our opinion, these divergent results could be induced by differences in vine sampling, cultivar, training system and method used for foliar disease severity estimation.

The results from the single-plot samples showed a tendency for necrotic area to increase with the number of expressions of the foliar symptoms. In comparing with asymptomatic vines, Andreini *et al*. (2009) noticed a significant reduction in xylem flux before esca foliar symptoms appeared in Cabernet-Sauvignon vines expressing chronic form over a three-year period. The larger volume of necrosis in vines with the severe chronic form could explain the reduction in xylem flux. Nevertheless, other factors are most likely involved. Christen *et al*. (2007) suggested that the esca foliar symptoms are not only caused by deficiencies in water transport through the defective xylem vessels but that phytotoxic compounds should be involved.

3. Logistic regression analysis : the importance of WR

For decades, it has been reported that white-rotted wood, also called wood decay or « amadou », is associated with esca (Larignon and Dubos 1997, Mugnai *et al.,* 1999, Ravaz 1898 ; Surico *et al.,* 2005 ; Viala, 1926). However, to our knowledge the logistic regression analysis reported in our study demonstrated for the first time that WR was the best indicator of the chronic form. The amount of WR in the vines significantly increased the probability of chronic form expression. Our fungal isolation results were consistent with those of the literature that indicate that WR is generally associated with the presence of *F. mediterranea* (Fischer 2009, Péros *et al.,* 2008) in Europe, including France. *F. mediterranea* is considered as the main pathogen causing WR in grapevine (Fischer 2006;Surico *et al.,* 2005) and in other woody plants such as citrus (Elena *et al.,* 2006), hazelnut (Pilotti *et al.,* 2010), and kiwifruit (di Marco *et al.,* 2004). In each case it causes tree decline. As for other WR fungi, it produces extracellular wall degrading enzymes including laccase and peroxidase (Abou-Mansour *et al.,* 2009 ; Mugnai *et al.,* 1999). The inoculation of vineyard-grown adult plants with *F. mediterranea* induces the production of necroses in living tissues (Sparapano *et al.,* 2000b) and it develops in the pith or heartwood tissues (Laveau *et al.,* 2009, Sparapano *et al.,* 2000b), but it does not induce foliar symptoms on plants. Although secondary metabolites from *F. mediterranea* have been characterised

(Tabacchi *et al.,* 2000), their roles in pathogenesis and in foliar esca expression are still relatively unknown. Moreover, chronic form of esca was also observed in young vines without the presence of *F. mediterranea*. Therefore the role of this fungus in triggering chronic form symptoms remains to be clarified.

The important area of necrotic wood in the cordons, close to the pruning wounds, suggested that wounds could serve as "open doors" for the pathogens. If confirmed, this highlights the importance of protecting the wounds and/or adapting the training system, for example, by using the Guyot-Poussard training system recommended by Geoffrion and Renaudin (2002). In this system, conceived by Poussard, unilateral wounds are generated. The spur is on the same side of the stick, so the wound size of the stick-annual removal is always above the cordons. This type of pruning makes the sap flow opposite to pruning wounds.

CONCLUSIONS

To better evaluate the severity of internal necroses in relation with esca disease, it is essential to take into account the amount and location of necroses relative to the functional woody tissues. We demonstrated that vines with acute foliar and chronic forms of esca can be characterised by the development of specific necroses. In addition, vines with the acute foliar form significantly differed from the other vines, showing very advanced peripheral tissue degradation in the xylem and cambial zones. For the chronic form of esca, a logistic model indicated that WR in the cordons was the best descriptor. The quantitative approach of vine internal necroses studied here would open up new strategies to understand the development of esca epidemic.

Acknowledgements: This study was funded by the Conseil Interprofessionnel des Vins de Bordeaux and the French National Wine Body (VINIFLHOR). We wish to thank all the vine-growers who participated in this study. We also thank J.M. Liminana for excellent technical assistance with image analysis.

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