SYMPTOMS AND FUNGI ASSOCIATED WITH DECLINING MATURE GRAPEVINE PLANTS IN NORTHEAST SPAIN

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SUMMARY

A field survey was carried out in Catalonia (northeast Spain) to characterize the decline of mature grapevines. The relationships of both external and internal symptoms of diseased plants and their associated mycoflora were studied. Co-occurrence of different internal disease symptoms was frequently observed, since 44% of the sampled plants had wood lesions commonly associated with at least two of the following decline diseases: eutypiose, black dead arm or esca. The results obtained suggest that apoplexy might not be associated only with esca-affected plants, since 60% of surveyed vines showing apoplexy showed also V-shaped necroses, which are commonly associated with eutypiose and black dead arm, and 20% were exclusively affected by V-shaped necroses. An experiment was conducted to establish the pathogenicity of the most representative fungi isolated from diseased tissues of declining plants, by artificially inoculating 1-year-old vines of cvs Macabeo and Tempranillo. As indicated by the extension of the vascular lesions, pathogenicity was confirmed for most of the species tested, namely *Botryosphaeria dothidea*, *Diplodia seriata*, *Eutypa lata*, *Neofusicoccum luteum*, *N. parvum* and *Phaeomoniella chlamydospora*.

Key words: black dead arm, esca, eutypiose, grapevine decline, phytopathogenic fungi, *Vitis vinifera*.

INTRODUCTION

The area given over to grapevine in Spain is about 1.2 M ha, which makes this country the leading one in the world for grape-growing, the third for wine production, and the second for raisin production (Anonymous, 2006; OIV data 2005, retrieved from http://www.oiv.org). Although *ca.* 97% of the total Spanish grapevine area is destined for wine production (Anonymous, 2006), thus viticulture is an essential component of the agricultural sector, grapevine declines and their associated pathogens are poorly known in this country. In the last decade, studies on diseases and pathogenic mycoflora associated with rootstocks (Aroca *et al.*, 2006), young vines (Armengol *et al.*, 2002; Giménez-Jaime *et al.*, 2006), and mature vines (Armengol *et al.*, 2001a, 2001b; Úrbez-Torres *et al.*, 2006a) have been carried out, but further studies are needed for a better insight in these complex diseases and an adequate estimation of their economic impact. Main decline diseases of mature grapevine observed in Spain include esca, eutypiose, and black dead arm (BDA), as reported by Armengol *et al.* (2001a, 2001b) and Úrbez-Torres *et al.* (2006a).

Esca is a complex disease where symptoms and their expression over time are highly variable (Mugnai *et al.*, 1999; Surico *et al.*, 2006). Two main types of esca episodes can be defined: the chronic esca and the acute syndrome, the latter also known as apoplexy (Mugnai *et al.*, 1999). Briefly, foliar symptoms of chronic esca are characterized by interveinal chlorosis or discolorations (yellowish in white cultivars and reddish in red cultivars) that later coalesce in large necrotic areas during summer. Vine apoplexy usually occurs in mid summer, when leaves of affected plants wither rapidly in a few days (Mugnai *et al.*, 1999). Despite of the external foliar symptoms of both esca types, several types of wood degradation have been described for esca, mainly including: (i) longitudinal brown streakings that appear as necrotic black spots in cross sections, (ii) pink-brown or dark red-brown necrotic areas, and (iii) wood decay. Many fungi have been reported to be involved in the esca syndrome; several Basidiomycetes species are responsible for the wood decay, with species in the genera *Fomitiporia*, *Fomitiporella*, and *Inocutis* (Fischer, 2006), while vascular necroses are caused mainly by *Phaeomoniella chlamydospora* and several *Phaeoacremonium* species (Surico *et al.*, 2006).

Eutypiose, also known as Eutypa dieback, is caused by the fungus *Eutypa lata* (Carter, 1988). The most recognized symptom of this disease is the stunted appearance of shoots at the early growth season, with small, cupped, and chlorotic leaves, and short internodes. Wood internal symptoms include characteristic V-shaped necroses when affected arms and trunks are cross-sectioned. Additionally, external cankers develop-

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ing from old pruning wounds can be observed.

BDA was first described by Lehoczky (1974), who associated this disease with *Botryosphaeria stevensii*. Several other species of Botryosphaeriaceae were later found in BDA-affected vines, the most frequent being *B. dothidea*, *Diplodia seriata* and *Lasiodiplodia theobromae* (Larignon *et al.*, 2001; van Niekerk *et al.*, 2006). Wood symptoms of BDA include V-shaped necroses, similar to those caused by *E. lata*, and longitudinal brown streakings along the affected tissues. Occasional stunted growth in early season was also reported for diseases caused by Botryosphaeriaceae species (Castillo-Pando *et al.*, 2001; Taylor *et al.*, 2005), thus resembling the symptoms caused by *E. lata*.

BDA foliar symptoms are controversial. While Lehoczky (1974) reported a slight diffuse chlorosis followed by leaf wilting, Larignon and Dubos (2001) described an early red or yellow-orange patchy discoloration of the leaves (in red- and white-berried grape varieties, respectively) that later developed into large marginal and interveinal necroses. However, Lecomte *et al.* (2005) and Surico *et al.* (2006) have shown and discussed the similarity between these late BDA-associated foliar symptoms and those typical of esca.

Additional more comprehensive information on the above diseases can be found in Carter (1988, 1991), Larignon *et al.* (2001), Lecomte *et al.* (2005), Mugnai *et al.* (1999), Surico *et al.* (2006) and van Niekerk *et al.* (2006).

The present study aims at characterizing the decline of mature grapevines in Catalonia, northeastern Spain, by determining the relationship of both external and internal symptoms of diseased plants with the existing mycoflora, and establishing the pathogenicity of fungi isolated from diseased tissues of declining plants.

MATERIALS AND METHODS

Field survey. Seventy-nine vineyards known to be affected by decline diseases from previous field surveys were visited between 2003 and 2005 in Catalonia. Field data and plant samples were collected each year from May to August. Eighteen grapevine varieties and three rootstocks were surveyed, white-berried cultivars included Chardonnay, Chenin Blanc, Garnatxa Peluda, Macabeo, Parellada, Sauvignon Blanc, White Grenache, and Xarel·lo, whereas red-berried cultivars were Cabernet Sauvignon, Carignane, Merlot, Pinot Noir, Red Grenache, Syrah, and Tempranillo, plus three unknown varieties and three rootstocks (110R, 140Ru and SO4). A total of 192 vines showing decline symptoms were surveyed from over 1500 inspected plants (about 20 decline-affected plants being examined per vineyard). Two to four affected plants were chosen from each vineyard for a careful symptom examination and wood sampling. Declining vines were examined visually and the nature of the external symptoms was annotated and attributed to known diseases: eutypiose, BDA or esca. Vines which showed stunted shoot growth in late spring, and V-shaped wood necroses were classified as affected by eutypiose/BDA during the field survey, since both diseases show similar symptoms (Castillo-Pando *et al.*, 2001; Taylor *et al.*, 2005). BDA foliar symptoms occurring in summer, as described by Larignon and Dubos (2001), were not considered as these symptoms could be confused with those of esca (Lecomte *et al.*, 2005; Surico *et al.*, 2006). Vines with characteristic interveinal chloroses and necroses, wood decay and vascular necroses different from V-shaped ones were classified as esca-affected plants. Vines with sectorial necrosis and either one of the esca-associated wood necroses were classified as affected by eutypiose/BDA and esca. Plants affected by apoplexy whether partial (1 to several arms) or total (whole plant) were considered as a separate class from the above diseases. Sections of trunks and arms, as well as the whole plant when appropriate, were taken to the laboratory for further examination and to conduct fungal isolations.

Fungal isolation and identification. Cross and longitudinal sections of arms and trunks of diseased vines were carefully examined and the type of wood necrosis was recorded. Four types of wood alteration were considered: V-shaped necroses, irregular central necroses, black spots shown in cross sections, and wood decay (Fig. 1, c to h). Wood pieces with each type of necrosis (approximately 10 cm in length) were excised every 20 cm of affected arms and trunks, and processed separately to isolate the fungi. Wood chips (about 5x5x5 mm; minimum 15 pieces per sample and type of necrosis) were surface-sterilized (3-4 min in 70% ethanol), blotted on sterile filter paper, plated onto potato dextrose agar (PDA, Difco, USA) amended with sulphate streptomycin (Sigma-Aldrich, USA) at 100 units/ml (Johnston and Booth, 1983) and incubated at 25ºC in the dark. When necessary, sporulation was induced by incubating fungal colonies in water agar with sterilized grapevine wood chips at 25ºC and under near-UV light/darkness for 12/12 h. Representative fungal isolates were maintained at 4ºC in sterile distilled water tubes with mycelial plugs.

Isolated fungi were identified on the basis of morphological characters of the colonies and reproductive structures. Identification was confirmed by analysing the DNA sequences from selected regions: the internal transcribed spacers ITS1 and ITS2 flanking the 5.8S rRNA gene (ITS), and parts of the translation elongation factor 1-alfa (EF1-α) and the β-tubulin genes, when applicable. Procedures of DNA extraction were as described by Alves *et al.* (2004), and PCR amplifications were done according to Alves *et al.* (2004) (ITS, for all fungi); Phillips *et al.* (2005) (EF1-α, for Botryosphaeriaceae species), and

Mostert *et al.* (2006) (β-tubulin, for *Phaeoacremonium* spp. and Botryosphaeriaceae species). DNA was sequenced as described by Alves *et al.* (2004). All regions were sequenced in both strands to clarify any nucleotide ambiguous position. BLAST searches in GenBank showing high identity levels with reference sequences (>97%) were used to confirm the identifications.

Pathogenicity tests. Twenty-eight isolates representing 11 fungal taxa were chosen for pathogenicity trials, for which several isolates (2 to 5) were selected for each of the species that were more frequently encountered in the field survey (Table 4). Artificial inoculations were conducted in May 2004 on 1-year-old grapevine plants of cvs Macabeo (white) and Tempranillo (red) cultivars grafted onto Richter 110 rootstocks. Plants were maintained in 3 liter pots filled with a 6:1 sand:peat mixture (Floratorf, Floragard, Germany) and watered regularly in a greenhouse. Plants were fertilised every two weeks with 10 ml of double-strength Hoagland-Arnon's solution (Hoagland and Arnon, 1950). Pathogenicity tests were performed in a completely randomized experimental design, with 18 inoculated plants per cultivar and isolate. A superficial wound (15 x 5 mm, reaching into the xylem) was made on the stem of each plant with a sterilized scalpel, 10 cm above the graft union. A mycelial plug (5 mm diameter) obtained from the margin of a fungal colony was placed in the wound with the mycelium facing the stem, and the wound was wrapped with Parafilm® (Pechiney Plastic Packaging, USA). Control plants were inoculated with sterile PDA plugs instead of the fungal inoculum.

Nine months after inoculation, the length of the internal vascular lesions was recorded, by removing the bark from the stem and measuring the necrotic lesions upwards and downwards the inoculation site. Surface sterilized wood pieces taken from necrotic tissues were plated on PDA to reisolate inoculated fungi so as to fulfill Koch's postulates. The length of necroses was used as an indicator of pathogenicity and was analyzed using ANOVA with the aid of the SPSS v.10 statistical package (SPSS Inc., USA), with 'grapevine variety' and 'isolate' as independent factors. After ANOVA, mean values of each treatment (isolate) were compared against their respective controls with the Dunnett two-tailed test. Additional ANOVA followed by Tukey's test were used to detect differences among isolates within a given species.

RESULTS

Field survey. A total of 192 diseased plants belonging to 18 different grapevine varieties and three different rootstocks were visually analysed and sampled for laboratory analyses. The most surveyed white-berried varieties were the local cvs Macabeo (56 vines), Xarel·lo (24) and Parellada (11), whereas the red-berried varieties included Tempranillo (30), Red Grenache (17), Cabernet Sauvignon (14), and Carignane (11). As to the remaining varieties and rootstocks, no more of 5 vines per source were checked. According to the external symptoms observed in the field, 58% of the surveyed vines were diagnosed as affected by eutypiose/BDA, 19% by esca, and 14% by apoplexy. The remaining cases included dead plants (5%), uncertain diagnosis (1%), and vines showing both eutypiose/BDA and esca symptoms (3%).

Internal symptoms appeared to be the result of multiple diseases that frequently coexisted in the same vine as shown in Table 1 and Fig. 1g, h. Of all sampled plants, 44% (84 vines) showed internal symptoms typi-

Table 1. Percentage of declining grapevine plants showing different internal symptoms for a given external symptomatology.

External symptoms	Number of vines	Internal symptoms
Eutypiose/BDA	111	40% V-shaped necrosis
		22% Black spots, central necroses, wood decay
		38% V-shaped necrosis, black spots, central necroses, wood decay
Esca	37	7% V-shaped necrosis
		37% Black spots, central necroses, wood decay
		56% V-shaped necrosis, black spots, central necroses, wood decay
Eutypiose/BDA + Esca	5	100% V-shaped necrosis, black spots, central necroses, wood decay
Apoplexy	26	20% V-shaped necrosis
		20% Black spots, central necroses, wood decay
		60% V-shaped necrosis, black spots, wood decay

cal of each of the three main grapevine diseases. Moreover, 38% of vines with typical external symptoms of eutypiose/BDA (42 vines) showed also internal symptoms of esca, 56% of vines with external symptoms of esca (21 vines) showed the typical V-shaped necrosis of eutypiose/BDA as well as internal esca symptoms, and 60% of the apoplectic vines (16 vines) showed internal symptoms of both eutypiose/BDA and esca (Table 1). In 19 vines (10% of the sampled plants), the internal symptoms did not match the outward aspect of the disease. Two of these vines showing external esca symptoms were free from internal wood lesions, whereas the remaining 17 plants showed stunted growth but only esca symptoms internally.

Forty-five percent of the plants showing external symptoms of eutypiose/BDA exhibited at least two different kinds of internal lesions (Table 2). The incidence of two or more concomitant internal symptoms of esca, eutypiose/BDA+esca, and apoplexy was even higher, namely 80%, 100% and 81%, respectively (Table 2). On the whole, an average of 63% of surveyed vines (121 vines) showed at least two different types of internal lesions.

Fungal isolation and identification. Isolations were attempted from 657 samples with V-shaped necroses, 314 with black spots, 297 with other necroses and 187 with wood decay, yielding 502 fungal isolates, i.e. 236 from V-shaped necroses, 104 from black spots, 97 from other necroses and 65 from wood decay (Table 3). Fungi were more frequently isolated from arms than trunks, as shown by the number of taxa isolated from each plant part and the number of isolates per taxon.

Diplodia seriata and *E. lata* were predominantly isolated from the V-shaped necroses, with 44.9% and 23.3% of the isolations made from arms and trunks, respectively (Table 3). However, the remaining species of Botryosphaeriaceae (*Neofusicoccum luteum*, *N. parvum*, *N. vitifusiforme*, *Dothiorella viticola*, and other unidentified *Botryosphaeria* spp) accounted for an additional 10.6% of isolations from arms and trunks. A small number of isolates of *E. lata* and Botryosphaeriaceae species originated from other lesion types. *Phaeomoniella chlamydospora* was mostly isolated from the black spots, and was identified in 73.1% of all isolations (Table 3). Several fungal species were associated with central necroses, with no single species clearly predominant. The fungi most frequently associated with these lesions were *Pa. chlamydospora* (24.7%), *D. seriata* (20.6%), *Pm. aleophilum* (12.4%), and *E. lata* (10.3%). Additionally, about 10 more taxa were isolated from central necroses, but with a low frequency (Table 3). *Fomitiporia mediterranea* was predominant in decayed wood and was identified in 53.8% of the cases (Table 3). Other fungi (e.g. *D. seriata*, *E. lata* and *Pa. chlamydospora*) were isolated from decayed wood with a low frequency.

Pathogenicity tests. Control plants of both grapevine cultivars grew normally during the experimental period. Wounds of control plants healed successfully although some vascular discolourations were noticed (Table 4). Isolations from control plants were negative. Only *N. luteum* and *N. parvum* caused wilting in a variable number of inoculated vines (Table 4). While *N. luteum* caused wilting of ten 'Tempranillo' and five 'Macabeo' plants, *N. parvum* caused a higher proportion of wilted 'Macabeo' than 'Tempranillo' vines. No additional foliar symptoms were observed among the remaining inoculated plants that could be related to a potential pathogenic effect of the tested isolates.

ANOVA showed the significance of the factors 'isolate' and 'grapevine variety' and their interaction (all $P<0.01$) on necrosis length. In general, necroses induced in 'Tempranillo' by each inoculated isolate were longer than in 'Macabeo' ($P<0.05$), but the differences between varieties were not significant for isolates denoted *Botryosphaeria dothidea* 353, *Cryptovalsa ampelina* 476, *D. viticola* 412, *E. lata* 481, *F. mediterranea* I-62, and *N. parvum* 434 and 444. However, significant differences between isolates and their respective controls were more frequent among 'Macabeo' vines. This was

Table 2. Percentages of declining grapevine plants showing different lesion types.

External symptoms[a]	Number of different internal lesion types[b]				
	0	1	2	3	4
Eutypiose/BDA	2	53	30	12	3
Esca	3	16	52	24	5
Eutypiose/BDA+Esca	0	0	20	80	0
Apoplexy	0	19	46	19	16

[a]Summary of observed external symptoms: Eutypiose/BDA: stunted appearance of shoots at the early growth season, with small, cupped, and chlorotic leaves, and short internodes. Esca: interveinal chlorosis or discolorations (yellowish in white cultivars and reddish in red cultivars) eventually with leaf marginal or interveinal necrosis. Apoplexy: drastic leaf withering, whether partial (1 to several arms) or total (whole plant).
[b]Sum of row percentages = 100. Lesion types may include one or more of the following internal symptoms in the same plant: V-shaped necrosis, irregularly-shaped necrosis around the pith, black spots, wood decay.

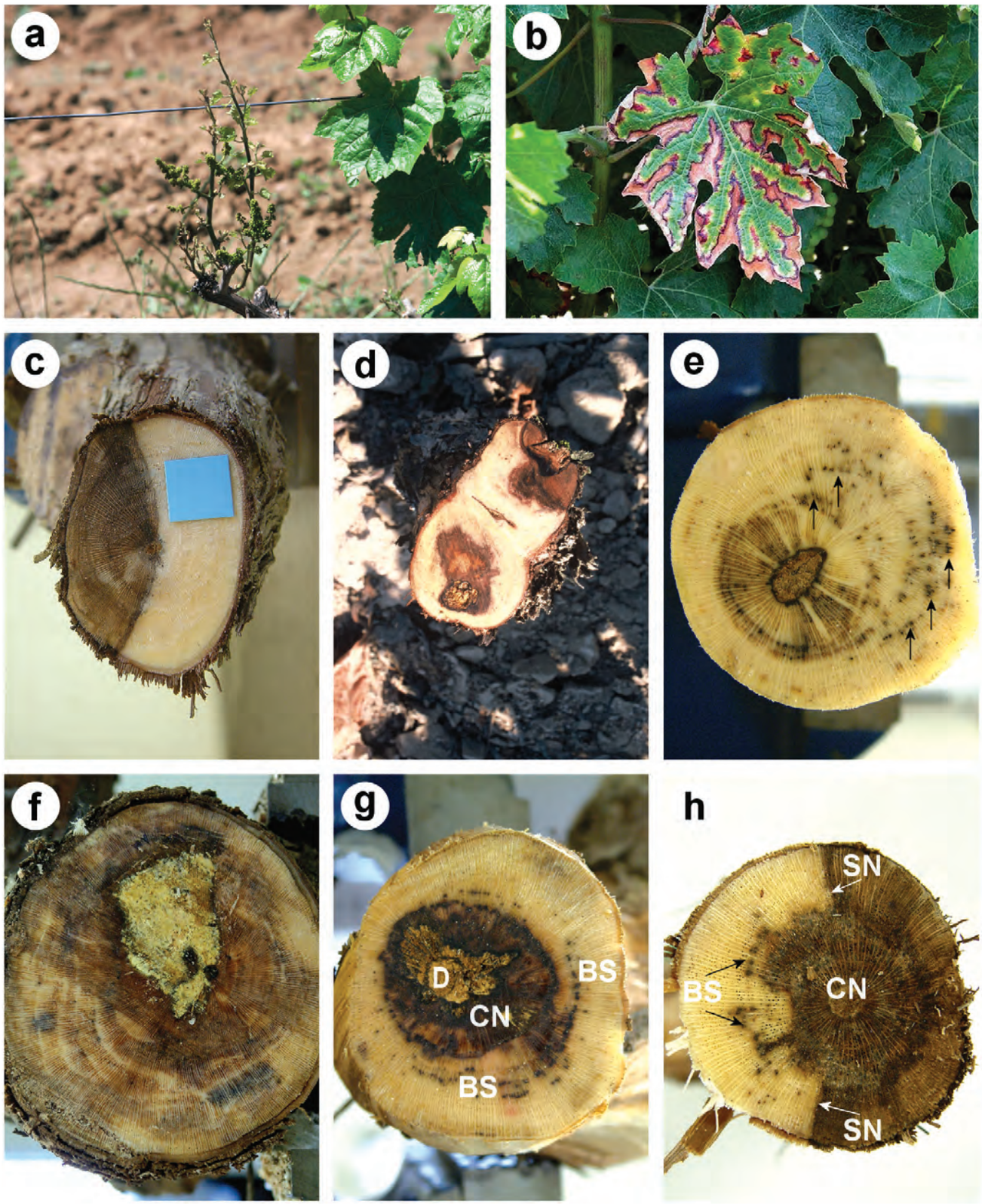

Fig. 1. Most common symptoms associated with grapevine declines: a. Reduced growth, and small, chlorotic leaves; b. Foliar symptoms of esca in a red grape cultivar, characterised by the interveinal necroses and chloroses; c. V-shaped necrosis. d. Irregularly-shaped necrosis around the pith (central necrosis); e. Black spots (shown by arrows), corresponding to vascular necroses; f. Wood decay, characterised by a yellowish soft tissue; g and h. Co-occurrence of internal symptoms; BS, black spots, CN, central necrosis, D, wood decay, SN, V-shaped necrosis. Symptoms "a" and "c" are usually associated with eutypiose and black dead arm, whereas symptoms "b" and "d" to "f" are associated with esca.

Table 3. Number of fungi isolated from wood lesions of declining grapevines.

Plant organ	Fungal species	Lesion type[a]				Total
		V-shaped necroses	Black spots	Central necroses	Wood decay	
Arms	*Acremonium* sp.	0	0	1	1	2
	Botryosphaeria dothidea	0	0	1	0	1
	Cryptovalsa ampelina	0	0	1	0	1
	Cylindrocarpon liriodendri	0	0	1	0	1
	Diplodia seriata	91	8	17	5	121
	Dothiorella viticola	1	0	0	0	1
	Eutypa lata	47	1	8	3	59
	Eutypa leptoplaca	1	0	0	0	1
	Eutypella vitis	1	0	0	0	1
	Fomitiporia mediterranea	3	1	2	19	25
	Fusarium spp.	3	0	3	1	7
	Neofusicoccum parvum	11	1	3	0	15
	Phaeoacremonium aleophilum	2	4	11	1	18
	Phaeoacremonium viticola	0	0	1	0	1
	Phaeoacremonium sp.	1	0	0	0	1
	Phaeomoniella chlamydospora	11	61	19	4	95
	Phoma-like sp.	1	2	0	0	3
	Phomopsis spp.	7	0	0	0	7
	Stereum hirsutum	0	1	0	0	1
	Unidentified Botryosphaeriaceae	6	0	1	0	7
	Unidentified Diatrypaceae	1	0	1	1	3
	Unidentified species	8	6	6	2	22
Trunk	*Acremonium* sp.	0	1	0	0	1
	Botryosphaeria dothidea	0	1	0	0	1
	Cryptovalsa ampelina	3	0	0	1	4
	Cylindrocarpon liriodendri	0	0	1	0	1
	Diplodia seriata	15	0	3	3	21
	Eutypa lata	8	1	2	2	13
	Fomitiporia mediterranea	0	0	3	16	19
	Fusarium spp.	1	0	3	0	4
	Neofusicoccum luteum	1	0	0	0	1
	Neofusicoccum parvum	5	1	0	0	6
	Neofusicoccum vitifusiforme	1	0	0	0	1
	Phaeoacremonium aleophilum	1	0	1	0	2
	Phaeoacremonium sp.	1	0	0	0	1
	Phaeomoniella chlamydospora	4	15	5	4	28
	Phomopsis spp.	0	0	1	0	1
	Unidentified Botryosphaeriaceae	1	0	1	0	2
	Unidentified species	0	0	1	2	3
Totals		236	104	97	65	502

[a]Number of lesions examined for isolations: V-shaped necroses, 657; black spots, 314; central necroses, 297; wood decay, 187.

probably due to the longer necroses (P<0.01) observed in the 'Tempranillo' control (1.4 cm), which were twice as long as those in 'Macabeo'. The most severe lesions were caused by *N. parvum* (necroses up to 12.7 cm long in 'Macabeo' and 13.8 cm in 'Tempranillo') and *N. luteum* (8.6 cm and 8.2 cm in the two cultivars, respectively). Other fungi that caused significant lesions in both cultivars were *Pa. chlamydospora* (2.5 to 5.5 cm), *E. lata* (1.3 to 4.0 cm), *D. seriata* (0.8 to 3.6 cm), and *B. dothidea* (3.2 cm in both cvs.) (Table 4). *F. mediterranea*, *Pm. aleophilum* and *C. ampelina* only caused significant necroses in 'Macabeo'. In average, lesions induced by these fungi were never longer than 2 cm in 'Macabeo' and 2.5 cm in 'Tempranillo' (Table 4). The isolates *D.*

seriata 421, *F. mediterranea* 356 and *Phomopsis* sp. 459 did not cause any significant lesion on any cultivar.

Fungal species with multiple isolates tested for pathogenicity showed some variability in the length of the lesions they caused. Thus lesions with a wide range of size were induced by *N. parvum* (maximum mean values about 2-3 fold greater than the minimum values), *D. seriata* (2-3 fold), and *E. lata* (1.5-2 fold), whereas less variation was observed for *C. ampelina*, *F. mediterranea*, *Pa. chlamydospora* and *Pm. aleophilum* (Table 4). Significant differences in the length of necroses were only observed among isolates of *D. seriata* and *N. parvum* (Table 4).

Reisolations from inoculated vines were successful for all of the inoculated fungi, although percentages of positive reisolations were variable among the fungal species (Table 4). In general, reisolations from 'Macabeo' plants were higher than those from 'Tempranillo'. Additionally, reisolation percentages were generally higher for the most virulent fungi (those causing longer necrosis, e.g. Botryosphaeriaceae species, and *Pa. chlamydospora*) than those from weak pathogens (e.g. *C. ampelina* and *F. mediterranea*).

Table 4. Number of wilted plants, length of vascular necroses and percentage of fungal recovery from grapevine plants (n=18) of cvs Macabeo and Tempranillo inoculated with selected fungi isolated from declining grapevines.

Fungal species	Isolate	'Macabeo'			'Tempranillo'		
		Wilted plants	Necrosis (cm)[a]	Fungal recovery (%)	Wilted plants	Necrosis (cm)[a]	Fungal recovery (%)
Botryosphaeria dothidea	353	0	**3.2**	100	0	**3.2**	100
Diplodia seriata[b]	398	0	**2.4** a	100	0	**3.5** a	67
D. seriata	421	0	0.8 c	83	0	1.9 b	28
D. seriata	I-29	0	**1.6** b	94	0	**3.0** ab	78
D. seriata	I-50	0	**1.5** b	100	0	**3.6** a	83
Dothiorella viticola	412	0	**1.7**	61	0	2.1	39
Cryptovalsa ampelina	413	0	**1.5**	50	0	2.5	39
C. ampelina	476	0	**1.7**	28	0	2.0	17
Eutypa lata	401	0	**1.7**	100	0	**2.9**	67
E. lata	411	0	**1.9**	94	0	**4.0**	50
E. lata	427	0	**1.3**	83	0	**3.6**	61
E. lata	438	0	**1.4**	100	0	2.3	50
E. lata	481	0	**2.0**	88	0	2.2	39
Fomitiporia mediterranea	356	0	0.7	33	0	1.5	11
F. mediterranea	452	0	**1.4**	33	0	2.5	22
F. mediterranea	I-62	0	**1.1**	18	0	1.4	17
Neofusicoccum luteum	519	5	**8.6**	100	10	**8.2**	100
Neofusicoccum parvum[b]	387	0	**4.0** c	94	0	**5.6** b	100
N. parvum	396	5	**10.8** a	100	1	**13.8** a	78
N. parvum	434	5	**12.7** a	100	0	**11.6** a	100
N. parvum	444	1	**6.7** b	100	0	**6.9** b	94
Phaeoacremonium aleophilum	449	0	**1.1**	67	0	2.4	83
Pm. aleophilum	477	0	**1.2**	83	0	2.2	100
Pm. aleophilum	I-10	0	**1.4**	89	0	1.9	94
Phaeomoniella chlamydospora	454	0	**2.5**	89	0	**4.5**	67
Pa. chlamydospora	I-8	0	**2.5**	78	0	**5.3**	89
Pa. chlamydospora	I-64	0	**2.5**	83	0	**5.5**	89
Phomopsis taxon 1	459	0	1.0	89	0	1.8	89
Control		0	0.7	0	0	1.4	0

[a]Values in bold are significantly different from the corresponding mean value of controls according to the two-tailed Dunnett's test.
[b]Mean values of necrosis lengths for *D. seriata* and *N. parvum* followed by different letters are significantly different according to the Tukey's test ($P<0.05$) within each species.

DISCUSSION

Results of field surveys confirmed the occurrence of the three main decline diseases of adult grapevines in Catalonia: eutypiose, BDA and esca. External and internal symptoms of both eutypiose and BDA, as recorded in May and June, looked very similar and thus were not reliable for distinguishing the two diseases, as it has been reported previously (Castillo-Pando *et al.*, 2001; Taylor *et al.*, 2005; Úrbez-Torres *et al.*, 2006b). Eutypiose and BDA could only be differentiated after isolation of the respective pathogens, *E. lata* and Botryosphaeriaceae spp. Moreover, in our study, late BDA symptoms, as described by Larignon *et al.* (2001a) (including leaf chlorosis and necrosis, leaf shedding, wilting of cluster, and brown streaking of the wood), were occasionally seen in plants affected by esca. Thus, external symptom expression of BDA-affected plants would need further investigation for a clear-cut identification of this disease. Since several species of Botryosphaeriaceae are associated with BDA (Larignon *et al.*, 2001; Surico *et al.*, 2006; van Niekerk *et al.*, 2006), it would be also interesting to investigate the pathogenic role of each of these fungi and the particular symptoms they cause in adult vines.

The present survey has also shown the frequent co-occurrence of internal symptoms associated with eutypiose, BDA and esca in the same plant, a condition already reported for esca and eutypiose (Mugnai *et al.*, 1999), although we were unable to find any quantitative example in the extant literature. The occurrence of multiple lesion types in the same plant in northeast Spain, which are especially frequent in the vine arms, may reflect multiple infection events through pruning wounds. In fact, it is widely accepted that most fungal pathogens associated with grapevine decline are airborne pathogens that penetrate the plant through wounds caused by the annual pruning (Carter, 1988; Mugnai *et al.*, 1999; Surico *et al.*, 2006; van Niekerk *et al.*, 2006).

Apoplexy is characterized by the sudden wilting and death in midsummer of vines or their organs, including clusters. Apparently healthy leaves may rapidly wilt basipetally and dry in a few days (Mugnai *et al.*, 1999). Weather conditions are thought to influence this phenomenon, since the apoplectic events often occur in hot summers, when rainfall is followed by dry, hot weather (Mugnai *et al.*, 1999). Apoplexy has been frequently described as a severe form of esca or specifically as an "acute esca syndrome" (Larignon and Dubos, 1997; Mugnai *et al.*, 1999; Graniti *et al.*, 2000; Surico, 2001; Surico *et al.*, 2006). However, the results of our study suggest that apoplectic events might not be restricted only to esca-affected plants, since a significant percentage of the surveyed vines showing apoplexy (60%; 15 vines) were also affected by V-shaped necroses, which are commonly associated with eutypiose and BDA (Carter, 1988; van Niekerk *et al.*, 2006). Moreover, 20% of apoplectic plants (5) had V-shaped necroses but no black spots, central necroses or wood decay, which are usually associated with esca. Mugnai *et al.* (1999) reported that both *D. seriata* and *E. lata*, which are often isolated from V-shaped necroses, can also be isolated from esca-affected plants, which supports our field observations. In this study, cross sections of arms and trunks of apoplectic vines showed a great percentage of dead, non-functional tissues. No quantitative data were recorded on the type and extension of these internal lesions. Further investigations are therefore needed to establish whether wood deterioration is related with apoplexy. Furthermore, water relationships of apoplectic plants should be studied to establish whether water stress could be related to apoplexy.

Fungal isolations of diseased wood showing a particular symptom indicated a general relationship between lesion type and isolated fungi. Thus, *D. seriata* and *E. lata* were mainly isolated from V-shaped necroses, *Pa. chlamydospora* from black spots, and *F. mediterranea* from decayed wood. Fungi isolated from central necroses included *D. seriata*, *Pa. chlamydospora* and *Pm. aleophilum*, which is in accordance with previous reports (Mugnai *et al.*, 1996; Larignon and Dubos, 1997; Mugnai *et al.*, 1999; Serra, 1999). However, some regional differences are observed in the distribution of some of these pathogens when our data are compared with those from neighbouring regions. In France, Larignon and Dubos (1997) isolated *E. lata* more frequently than any botryosphaeriaceous fungus from V-shaped necrosis, whereas our study and a previous one (Armengol *et al.*, 2001a) showed a greater incidence of *D. seriata* than *E. lata* in Spanish vineyards. Úrbez-Torres *et al.* (2006b) reported the same from California. It is suggested that *E. lata* is probably less abundant in dryer Mediterranean climate countries as compared with other cooler and rainy regions, since *E. lata* dispersion is enhanced when mean annual rainfall exceeds 350 mm (Carter, 1991; Mugnai *et al.*, 1999).

Most of the species tested for pathogenicity showed significant longer necrotic lesions than those in 'Macabeo' and 'Tempranillo' controls. Only *N. luteum* and *N. parvum* caused wilting of inoculated plants but no external disease symptoms were recorded for any other fungus-plant combination during the experimental period. Some influencing factors have been suggested to explain this phenomenon, which include the short experimental period, and other unsuitable experimental conditions such as the use of young, potted plants, and the inoculation of fungi into green, non-lignified plant tissues. Moreover, our pathogenicity tests were done using mycelium instead of spores as inoculum sources, which does not correspond to natural conditions for fungal infection. Additionally, it has been reported that some fungi (e.g. *F. mediterranea*) are only able to colonize grapevine tissues previously damaged by other fungi (Larignon and Dubos, 1997). A combination of the above factors could lead to unsuccesful fungal coloniza-

tion of inoculated plant tissues, as shown by the low recovery of some fungi (e.g. *C. ampelina*, *F. mediterranea*) and the short, non-significant necroses recorded occasionally in pathogenicity tests.

Lack of foliar symptom in plants artificially inoculated with known grapevine pathogens has occasionally been reported (Larignon and Dubos, 1997; Mugnai *et al.*, 1999). Since foliar symptom expression often fails to occur in artificial inoculations, the pathogenicity and virulence of fungi have often been evaluated analysing the necrotic lesions caused by fungi in the plant vascular tissues, as reported in previous works (Mugnai *et al.*, 1999; Van Niekerk *et al.*, 2004; Surico *et al.*, 2006).

Pathogenicity has been reported previously for several Botryosphaeriaceae species (van Niekerk *et al.*, 2004; Taylor *et al.*, 2005; van Niekerk *et al.*, 2006), *E. lata* (Carter *et al.*, 1985; Carter, 1991; Péros *et al.*, 1999; Sosnowski *et al.*, 2007), *F. mediterranea* (Sparapano *et al.*, 2001), and *Pm. aleophilum* and *Pa. chlamydospora* (Adalat *et al.*, 2000; Eskalen *et al.*, 2001; Sparapano *et al.*, 2001; Halleen *et al.*, 2007). *Neofusicoccum luteum* and *N. parvum* were the most virulent pathogens tested in our study. While *N. parvum* was proven to be a virulent pathogen by van Niekerk *et al.* (2004), pathogenicity of *N. luteum* seems controversial. Van Niekerk *et al.* (2004) considered this species as a low virulent pathogen since it caused no significant necroses on inoculated mature canes of cvs Chardonnay and Cabernet sauvignon in South Africa. However, in our study *N. luteum* was clearly pathogenic.

Pathogenicity of *D. seriata* has also been debated, as summarized by Úrbez-Torres *et al.* (2006b). This fungus was considered as a weak pathogen in Portugal (Phillips, 2002), but virulent in Chile (Auger *et al.*, 2004), South Africa (van Niekerk *et al.*, 2004) and Australia (Castillo-Pando *et al.*, 2001), although Taylor *et al.* (2005), in Australia, reported no significant vascular lesions caused by *D. seriata* to inoculated grapevine cuttings. In accordance with previous reports (Larignon *et al.*, 2001; van Niekerk *et al.*, 2004), our field observations and the results of pathogenicity tests indicate that *D. seriata* is pathogenic, though to a variable extent.

Eutypa lata is a widely-known pathogen of grapevine (Carter, 1988, 1991; Dubos, 1996). In our study, all five isolates tested for pathogenicity caused significant necrotic lesions on 'Macabeo' vines while those in 'Tempranillo' were significant only for three fungal isolates (401, 411 and 427). Although these findings may be indicative of variability in pathogen virulence, more isolates should be tested to confirm this hypothesis. Variability in virulence of *E. lata* has been shown previously (Péros *et al.*, 1999; Sosnowski *et al.*, 2007).

Our study confirmed the pathogenicity of *Pa. chlamydospora* although no foliar symptoms were observed during the experimental period. Foliar symptom expression due to infection by *Pa. chlamydospora* only were observed after long inoculation periods (2-3 years) in mature plants artificially inoculated with this pathogen (Sparapano *et al.*, 2001). Foliar symptoms of esca were reproduced on 'Thompson seedless' vines 6 months after inoculation with *Pm. aleophilum* and *Pa. chlamydospora* (W.D. Gubler, personal communication). The remaining fungal species tested in our study were considered as non-pathogenic (*Phomopsis* sp. taxon 1) or weakly pathogenic (*C. ampelina*, *F. mediterranea* and *Pm. aleophilum*), based on the size of the necrotic lesions caused. However, a pathogenic behaviour was reported for the latter two species (Adalat *et al.*, 2000; Eskalen *et al.*, 2001; Sparapano *et al.*, 2001).

Several fungal pathogens occur at the same time in the same grapevine, each one causing a particular wood lesion. This may lead to complex relationships among these pathogens and the host plant. This study has shown that co-occurrence of internal disease symptoms and their associated fungi is frequent in northeast Spain, and that the relationships between visual external symptoms and inferred internal lesions are often misleading. This makes field diagnosis of these wood diseases difficult when only the external symptoms are considered.

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