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

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Running title: Grapevine decline and associated pathogenic fungi

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Summary

A field survey was carried out in the Catalonia region of 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 frequent in Catalonia, since 44% of 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 also suggest that apoplexy might not be associated only with esca-affected plants, since 60% of surveyed plants showing apoplexy were also affected by 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 most representative fungi isolated from the diseased tissues of declining plants, by artificially inoculating 1-year-old plants of 'Macabeo' and 'Tempranillo' varieties. Pathogenicity of fungi, as indicated by vascular lesion extension, was confirmed for most of the species tested, namely *Botryosphaeria dothidea*, *Diplodia seriata*, *Eutypa lata*, *Neofusicoccum luteum*, *N. parvum* and *Phaeomoniella chlamydospora*.

Introduction

The area of vineyards in Spain consists of about 1,2 Mha, thus making Spain the leading country in grapevine culture in the world, the third in the world in wine production, and the second in raisin production (data from OIV, year 2005 [retrieved from Internet Oct. 15, 2008 at http://www.oiv.org]; Anonymous, 2006). Grapevines are widespread in Spain, where about 97% of the total grapevine area is managed for wine production (Anonymous, 2006). Although viticulture is an essential component of the agriculture sector in Spain, grapevine declines and their associated pathogenic fungi are poorly known in this country. In the last decade, studies on diseases and pathogenic mycoflora associated to rootstocks (Aroca *et al.*, 2006), young vines (Armengol *et al.*, 2002; Giménez-Jaime *et al.*, 2006), and mature vines (Armengol *et al.*, 2001a, b; Úrbez-Torres *et al.*, 2006a) have been carried out in Spain, but further studies are needed to increase the knowledge regarding these complex diseases. Unfortunately, no quantitative data about the economic impact of these declines on Spanish grapevine production have been calculated. Main decline diseases of mature grapevine observed in Spain include esca, eutypiose, and black dead arm (BDA), as reported by Armengol *et al.* (2001a, b) 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 cross sections of affected arms and trunks are made. Additionally, external cankers developing from old pruning wounds can be observed. BDA was first described by Lehoczky (1974) who associated this disease with *Botryosphaeria stevensii*, but later several other species of Botryosphaeriaceae have been associated with the disease, including *B. dothidea*, *Diplodia seriata* and *Lasiodiplodia theobromae* as the most frequently related fungi (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. Stunted growth in early season has also been described occasionally 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 also a matter of controversy; while Lehoczky (1974) reported a slight diffuse chlorosis and an eventual later leaf wilting, Larignon and Dubos (2001) reported an early red or yellow-orange patching (in red and white grape varieties, respectively) that later develop 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 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 to characterize the grapevine decline of mature grapevines in the northeast Spanish region of Catalonia, by 1) determining the relationship of both external and internal symptoms of diseased plants with the existing mycoflora, and 2) establishing the pathogenicity in grapevine of fungi isolated from the 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, NE Spain. Field data and plant samples were collected each year from May to August. Eighteen grapevine varieties and three rootstocks were surveyed: the white varieties included Chardonnay, Chenin Blanc, Garnatxa Peluda, Macabeo, Parellada, Sauvignon Blanc, White Grenache, and Xarel·lo; the red varieties included Cabernet Sauvignon, Carignane, Merlot, Pinot Noir, Red Grenache, Syrah, and Tempranillo; three unknown varieties and three rootstocks (110R, 140Ru and SO4). A total of 192 vines showing decline symptoms were surveyed from over 1500 total inspected plants (about 20 decline-affected plants being examined per vineyard). Two to four affected plants per vineyard were chosen 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 diseased arms and trunks 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 seen in cross sections, and wood decay (see Figure 1, c to h). Pieces of each type of necrosis (approximate size 10 cm in length) were obtained every 20 cm of affected arms and trunks, and processed separately to isolate the fungi. Wood chips (about 5x5x5 mm; minimum n=15 pieces per sample and necrosis type) were surface-sterilized (3-4 min in 70% ethanol), blotted on sterile filter paper to remove excessive ethanol, and plated onto Potato Dextrose Agar (PDA, Difco Laboratories, Detroit, MI, USA) amended with sulphate streptomycin (Sigma-Aldrich Co., St. Louis, MO, USA) at 100 units per ml (Johnston and Booth, 1983). Plates were incubated at 25 ºC in darkness to get pure cultures. When necessary, sporulation was induced by incubating the fungal colonies in water agar with sterilized grapevine wood chips at 25 ºC and under near-UV light/darkness for 12/12 hours. Representative isolates were maintained at 4 ºC in sterile distilled water tubes with mycelial plugs.

Isolated fungi were identified on the basis of morphological characters of colonies and reproductive structures. Eventually, identification of isolates 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 done as described in Alves *et al.* (2004), and PCR amplifications were done according to the methods described by Alves *et al.* (2004) [ITS; for most of fungi], Phillips *et al.* (2005) [EF1-α; for Botryosphaeriaceae species] and Mostert *et al.* (2006) [β-tubulin; for *Phaeoacremonium* spp. and Botryosphaeriaceae species]. DNA sequencing was done as described by Alves *et al.* (2004). All regions were sequenced in both strands to clarify any nucleotide ambiguous position. BLAST searches at GenBank showing high identites with reference sequences (>97%) were used to confirm the identifications.

Pathogenicity test

Twenty-eight isolates representing 11 fungal taxa were chosen for the pathogenicity trial, where several isolates (2 to 5) were selected for species which showed to be more frequent after the field survey (Table 4). Artificial inoculations were conducted in May 2004 on 1-year-old grapevine plants of ‘Macabeo’ (white) and ‘Tempranillo’ (red) cultivars grafted onto Richter 110 rootstocks. Plants were maintained in 3 liter pots filled with a sand:peat mixture (6:1, v:v; Floratorf peat, from Floragard, Oldenburg, 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). The pathogenicity test was 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, Menasha, WI, 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 from the site of inoculation. Surface sterilized wood pieces taken from the stem necrotic tissues were plated on PDA to reisolate the inoculated fungi and thus fulfill Koch’s postulates. The length of necroses was used as an indicator of the pathogenicity of fungi. Necrosis lengths were analyzed using ANOVA with the aid of the SPSS v.10 statistical package (SPSS Inc., Chicago, IL, 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 varieties were the local cvs. 'Macabeo' (56 vines), 'Xarel·lo' (24) and 'Parellada' (11), whereas the red varieties included 'Tempranillo' (30), 'Red Grenache' (17), 'Cabernet Sauvignon' (14), and 'Carignane' (11). Frequencies for the remaining varieties and rootstocks were never over 5 plants. According to the external symptoms observed in the field, 58 % of the surveyed plants were diagnosed as affected by eutypiose/BDA, 19 % of plants were affected by esca, and 14 % by apoplexy. The remaining cases included dead plants (5 %), uncertain diagnoses (1 %), and plants combining both eutypiose/BDA and esca symptoms on the same individual (3 %).

Internal symptoms appeared to be the result of multiple diseases and were frequently co-occurrent, as shown in Table 1 and Figure 1.g, h. Forty-four percent of all sampled plants (n=84) showed internal symptoms characteristic of each of the three main grapevine diseases occurring in the same plant. Moreover, 38 % of plants with typical external symptoms of eutypiose/BDA (n=42) also showed internal symptoms of esca, 56 % of plants (n=21) with external symptoms of esca showed the typical V-shaped necrosis of eutypiose/BDA as well as internal esca symptoms, and 60 % of the apoplectic plants (n=16) showed internal symptoms of both eutypiose/BDA and esca (Table 1). In 19 plants (10 % of total sampled plants), the internal symptoms did not match with the external visual diagnosis of the disease. Two of these plants showing external esca symptoms were free from any internal wood lesion, whereas the remaining 17 plants showed stunted growth externally but only esca symptoms internally.

Forty-five percent of the plants observed with external symptoms of eutypiose/BDA showed at least two different kinds of internal lesions (Table 2). Percentages of two or more concomitant internal symptoms for esca, eutypiose/BDA+esca, and apoplectic plants were even higher, namely 80%, 100% and 81%, respectively (Table 2). In all, an average of 63% of surveyed plants (n=121) 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. Isolations yielded 502 fungal isolates: 236 isolates from V-shaped necroses, 104 from black spots, 97 from other necroses and 65 from wood decay (Table 3). Fungi were more frequently isolated in arms than in 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* species) 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 predominantly isolated from the black spots, and was identified in 73.1 % of all the isolations (Table 3). Several fungal species were associated with central necroses, with no single species clearly predominant. The most frequently associated fungi to 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 low frequencies (Table 3). *Fomitiporia mediterranea* was predominant in the decayed wood, as was identified in 53.8 % of the isolations (Table 3). Low frequencies of other fungi (e.g. *D. seriata*, *E. lata* and *Pa. chlamydospora*) were isolated from decayed wood.

Pathogenicity test

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 yielded no fungus. Only *N. luteum* and *N. parvum* caused the wilting of the plant in a variable number of inoculated plants (Table 4). While *N. luteum* caused wilting of ten 'Tempranillo' plants and five 'Macabeo' plants, *N. parvum* caused a higher proportion of wilted plants in 'Macabeo' than in 'Tempranillo'. 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 the necrosis lengths. In general, necrosis lengths recorded on 'Tempranillo' for each inoculated isolate were longer than for their equivalents in 'Macabeo' (P<0.05), but the differences between varieties were not significant for isolates *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 the 'Macabeo' plants. This was probably due to the longer necrosis (P<0.01) observed in the 'Tempranillo' control (1.4 cm), twice as long as those on '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, respectively). Other fungi that caused significant lesions in plants of both grapevine 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). *Fomitiporia mediterranea*, *Pm. aleophilum* and *C. ampelina* only caused significant necroses in 'Macabeo'; in general, mean lesion lengths for 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 being tested for pathogenicity showed some variability on the lesion lengths they caused. Thus, a wide range was observed for *N. parvum* (maximum mean values about 2-3 times greater than the minimum ones), *D. seriata* (2-3 times), and *E. lata* (1.5-2 times), whereas less variation was observed for *C. ampelina*, *F. mediterranea*, *Pa. chlamydospora* and *Pm. aleophilum* (Table 4). Only significant differences among isolates in the necrosis lengths for *D. seriata* and *N. parvum* were detected (Table 4).

Reisolations from the inoculated plants 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*).

Discussion

The results obtained during the field survey confirmed the occurrence of the three main decline diseases of adult grapevine plants in Catalonia: eutypiose, BDA and esca. External and internal symptoms of both eutypiose and BDA, recorded in May and

June, looked very similar and thus were not reliable to distinguish between these 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 were only differentiated after isolating the respective pathogens, *E. lata* and Botryosphaeriaceae spp, from the diseased tissues. Additionally, observations of late symptoms of BDA as described by Larignon *et al.* (2001) (including leaf chlorosis and necrosis, leaf fall, cluster wilting, and the occurrence of a brown streaking of the wood under the bark) were occasionally associated with plants affected by esca in this study. Thus, external symptom expression of BDA-affected plants would need further investigation to clearly identify this disease. Since BDA could be related with several species of Botryosphaeriaceae (Larignon *et al.*, 2001; Surico *et al.*, 2006; van Niekerk *et al.*, 2006), it would be also interesting to know the pathogenic role of each botryosphaeriaceous fungus and the particular symptoms it causes on adult plants.

Survey results also showed the high co-occurrence of internal symptoms associated with eutypiose/BDA and esca in the same plant. This has been reported for esca and eutypiose (Mugnai *et al.*, 1999), although we were not able to find any quantitative example in previous literature. The occurrence of multiple lesion types in the same plant in NE Spain, which were especially frequent in arms, may reflect different events of infections through the pruning wounds. It is widely accepted that most fungal pathogens associated with grapevine declines are airborne pathogens and penetrate into the plant through the annual pruning of shoots (Carter, 1988; Mugnai *et al.*, 1999; Surico *et al.*, 2006; van Niekerk *et al.*, 2006).

Apoplexy is characterized by the sudden wilting and death of vines or vine-parts including clusters in midsummer. Apparent healthy leaves rapidly wilt and dry basipetally 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 obtained in this study suggest that apoplectic events might not be restricted only to esca-affected plants, since a significant percentage of surveyed plants showing apoplexy (60 %; n=15) 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 (n=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*, often isolated from V-shaped necroses, are frequently 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 research is then needed to establish whether wood deterioration is related with apoplexy. Additionally, 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 the lesion type and the 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 several species, such as *D. seriata*, *Pa. chlamydospora* and *Pm. aleophilum*. In general, these results are 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 comparing our data with those obtained in neighbouring regions. In France, Larignon and Dubos (1997) isolated *E. lata* more frequently than any Botryosphaeriaceous fungi 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) also showed this for California. It is suggested that *E. lata* is probably less abundant in dryer Mediterranean climate countries as compared to 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 the 'Macabeo' and 'Tempranillo' controls. Only *N. luteum* and *N. parvum* caused the 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 a 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, pathogenicity tests in this study 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 previuosly damaged by other fungi (Larignon and Dubos, 1997). An unsuitable combination of the above factors could lead to the unsuccesful fungal colonization of the inoculated plant tissues, as seen on the low recovery of some fungi (e.g. *C. ampelina*, *F. mediterranea*) and the short, non-significant necroses recorded occasionally in the pathogenicity test. Lack of foliar symptom expression in artificially inoculated plants with known grapevine pathogens has been reported occasionally (Larignon and Dubos, 1997; Mugnai *et al.*, 1999). Since foliar symptom expression often fails to occur in artificial inoculations, pathogenicity and virulence of fungi have been often concluded from the analysis of 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 previously reported 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 ‘Chardonnay’ and ‘Cabernet Sauvignon’ varieties in South Africa. However, in our study *N. luteum* was clearly pathogenic.

Pathogenicity of *D. seriata* has been also disputed, as summarized by Úrbez-Torres *et al.* (2006b); it has been considered weakly pathogenic in Portugal (Phillips, 2002), but a virulent pathogen in Chile (Auger *et al.*, 2004), Australia (Castillo-Pando *et al.*, 2001) and South Africa (van Niekerk *et al.*, 2004). Additionally, Taylor *et al.* (2005) observed no significant vascular lesions caused by this fungus on inoculated grapevine cuttings in Australia. In our study, field observations of diseased tissues and the results obtained in the pathogenicity test would suggest that *D. seriata* is pathogenic. Additionally, the range observed in necrosis lengths also suggests variability in pathogen virulence. This is in accordance with previous reports (Larignon *et al.*, 2001; van Niekerk *et al.*, 2004).

Eutypa lata is a widely-known pathogen of grapevine (Carter, 1988; Carter, 1991; Dubos, 1996). In our study, all five isolates tested for pathogenicity on 'Macabeo' plants caused significant necrosis while those in 'Tempranillo' were significant only in three isolates (401, 411 and 427). These findings may indicate variability in pathogen virulence but 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). Pathogenicity of *Pa. chlamydospora* was also confirmed in this study, although no foliar symptoms were observed during the experimental period. Foliar symptom expression due to *Pa. chlamydospora* only have been observed after long inoculation periods (2-3 years) in mature plants artificially inoculated with this pathogen (Sparapano *et al.*, 2001). Foliar symptoms of esca have been reproduced on Thompson Seedless vines 6 months after inoculation with *Pm. aleophilum* and *Pa. chlamydospora* (W. D. Gubler, *pers. comm.*). The remaining species tested in our study were considered non-pathogenic (*Phomopsis* sp. taxon 1) or weakly pathogenic (*C. ampelina*, *F. mediterranea* and *Pm. aleophilum*) from the necroses they caused. However, pathogenic effects have been observed for the latter two species (Adalat *et al.*, 2000; Eskalen *et al.*, 2001; Sparapano *et al.*, 2001).

Concomitant fungal pathogens occur 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 are frequent in NE Spain, and that the relationships between visual external symptoms and inferred internal lesions often are misleading. This makes field diagnosis of the diseases difficult when only the external symptoms are considered.

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Table 1. Percentages of declining grapevine plants showing different internal symptoms for a given external symptomology.

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

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

External symptoms[2]	Number of different internal lesion types[1]				
	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

[1]: 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.

[2]: Summary of observed external symptoms: *Eutypiose*: 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).

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

Plant part	Species	Lesion type[1] V-shaped necroses	Black spots	Central necroses	Wood decay	Total
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

1: Numbers of lesions examined for isolations: V-shaped necroses: n=657; black spots: n=314; central necroses: n=297; wood decay: 187.

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

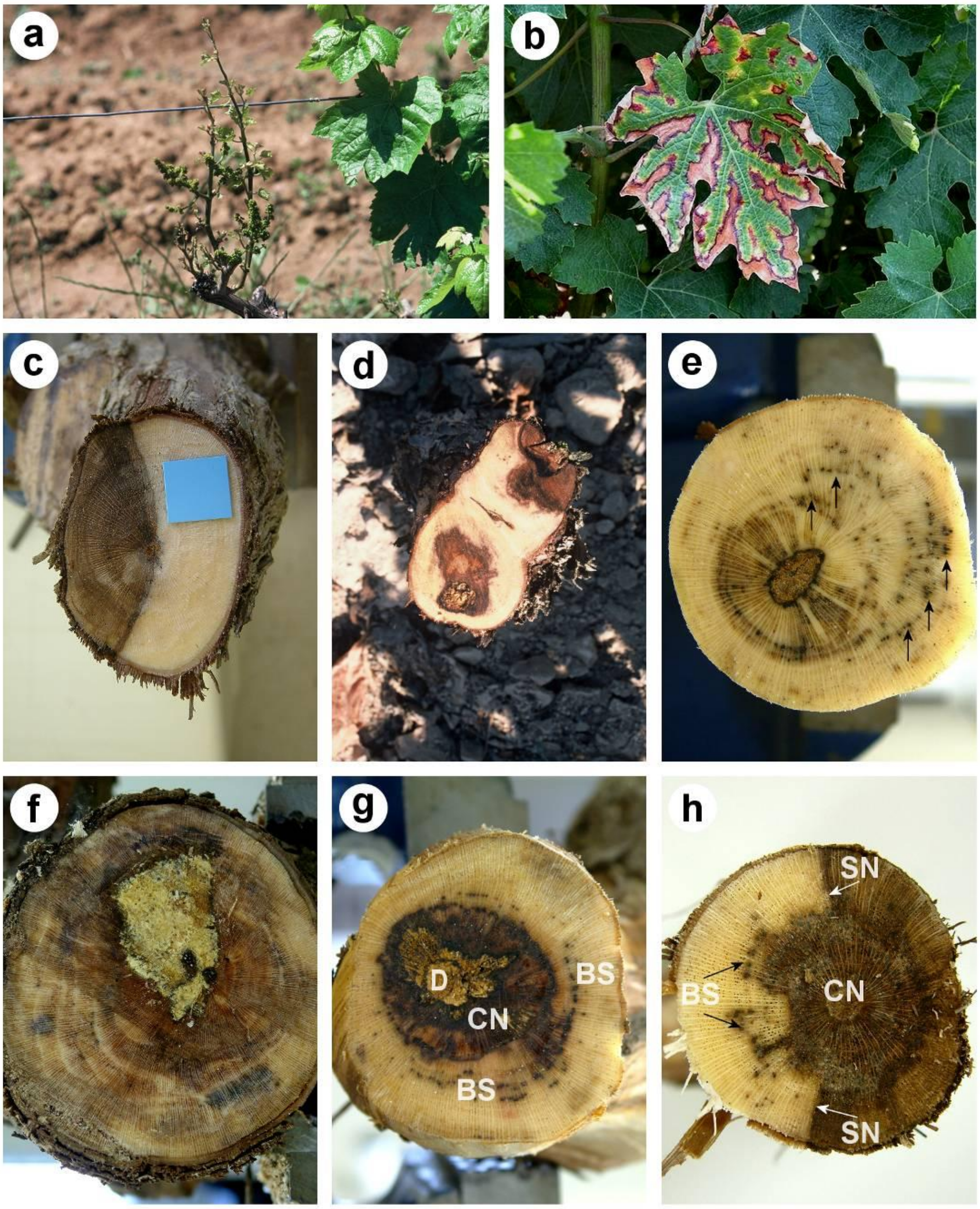
		Macabeo			**Tempranillo**		
Species	**Isolate**	Wilted plants	Necrosis (cm)[1]	% Myc. recovery	Wilted plants	Necrosis (cm)[1]	% Myc. recovery
Botryosphaeria dothidea	353	0	**3.2**	100	0	**3.2**	100
Diplodia seriata[2]	398	0	**2.4** a	100	0	**3.5** a	67
D. seriata	421	0	0.8 c	83	0	1.9 b	28
D. seriata	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[2]	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

[1]: Values in bold characters are significantly different from the corresponding control mean value according to the two-tailed Dunnett's test.

[2]: Mean values of necrosis lengths for *D. seriata* and *N. parvum* followed by different letters are different according to the Tukey's test ($P<0.05$) within each species.

Figure 1. Most habitual 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 to esca.

Figure 1



(low resolution image, for reviewing purposes only)