Instituto Nacional de Investigación y Tecnología Agraria y Alimentación (INIA)
Available online at www.inia.es/sjar

Spanish Journal of Agricultural Research 2008 6(4), 650-660
ISSN: 1695-971-X

Identification and characterization of fungi associated with esca in vineyards of the Comunidad Valenciana (Spain)

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Abstract

Grapevines sampled in the Comunidad Valenciana in Spain were examined for esca symptoms, and the different vine parts (trunk, cane and young vine) were surveyed for the presence of esca-related fungi. The fungal species most frequently identified were the mitosporic fungi *Diplodia mutila (Dmu), Phaeoacremonium aleophilum (Pal), Phaeomoniella chlamydospora (Pch)* and *Phomopsis viticola (Pvi)* and the basidiomycetes *Fomitiporia mediterranea (Fm)* and *Stereum hirsutum (Shi)*. PCR and sequencing using the universal pair primers ITS1-ITS4 confirmed the identification, these techniques proved very useful, particularly in cases where fruiting was not evident and/or pure cultures did not display enough diagnostic traits. *Dmu* and both basidiomycetes were the most frequent fungi associated with esca in this region. *Dmu* was the only fungal species found in all vine parts studied, being significantly more present in canes (82%) than in trunks (58%) or shoots (28%). Moreover, both basidiomycetes (50% *Fm* and 56% *Shi*) and *Pch* (32%) were most frequently found in trunks.

Additional key words: Basidiomycetes, grapevine, ITS, mitosporic fungi, molecular characterization, morphological characteristics, rDNA, *Vitis vinifera*.

Resumen

Identificación y caracterización de hongos asociados a la yesca en viñedos de la Comunidad Valenciana (España)

Se muestrearon viñas en la Comunidad Valenciana y se examinaron para detectar síntomas de yesca, y analizar la presencia de hongos relacionados con dicha enfermedad en las diferentes partes de la planta (tronco, caña y sarmiento). Las especies fúngicas identificadas de forma más frecuente fueron los hongos mitospóricos *Diplodia mutila (Dmu), Phaeoacremonium aleophilum (Pal), Phaeomoniella chlamydospora (Pch)* y *Phomopsis viticola (Pvi)* y los basidiomicetes *Fomitiporia mediterranea (Fm)* y *Stereum hirsutum (Shi)*. Dicha identificación fue confirmada mediante PCR y secuenciación con la pareja de primers universal ITS1-ITS4, siendo especialmente útil en aquellos casos donde la fructificación no fue evidente y/o en los cultivos puros que no mostraron suficientes caracteres diagnósticos. *Dmu*, junto con ambos basidiomicetes, fueron los hongos más encontrados asociados a la yesca en esta región. *Dmu* fue la única especie fúngica presente en todas las partes de la viña estudiadas, con una incidencia significativa en cañas (82%) comparada con los troncos (58%) y los sarmientos (28%). Además, se encontraron de forma mayoritaria en el tronco los basidiomicetes (50% *Fm* y 56% *Shi*), así como *Pch* (32%).

Palabras clave adicionales: Basidiomicetes, caracterización molecular, hongos mitospóricos, ITS, rDNA, viñedo, *Vitis vinifera*.

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Received: 07-09-07. Accepted: 07-10-08.

Abbreviations used: BLAST (basic local alignment search tool), *Dmu* (*Diplodia mutila), Fm (Fomitiporia mediterranea),* ITS (internal transcribed spacer), MA (malt agar), *Pal (Phaeoacremonium aleophilum), Pch (Phaeomoniella chlamydospora),* PDA (potato dextrose agar), *Pvi (Phomopsis viticola),* rDNA (ribosomic DNA), RT (room temperature), *Shi (Stereum hirsutum),* TAE (Tris-acetate-EDTA), UTM (universal transverse mercator), YPD (yeast peptone dextrose).

Introduction

Esca syndrome and other trunk diseases of fungal origin have become a growing threat to grapevines throughout the world (Dubos and Larignon, 1988) hampering the economic viability of vineyards everywhere (Morton, 2000). Grapevine decline symptoms in grape-growing regions of Spain have been reported in several surveys carried out in Spanish vineyards in the last few years (Armengol *et al*., 2001).

Esca is a disease complex displaying highly variable symptoms that can appear in severe (also called apoplexy) or mild form (Dubos and Larignon, 1988). Apoplexy is characterized by the sudden wilting and death of whole vines or vine-parts in midsummer (Mugnai *et al*., 1999). Mild symptoms are present inside the trunk and arms, on canes and vine shoots, leaves and berries. In affected trunks and arms, cross sections show a central necrotic and decayed area, in which sound wood gradually becomes spongy and soft, surrounded by a black line (Chiarappa, 1959).

Etiology of esca has been under study for over a century; nevertheless, taxonomic knowledge of several esca-associated microorganisms has been and still is controversial (Mugnai *et al*., 1999). In recent years, studies on the fungi associated with decline symptoms have focused on species identity.

To date, several *Phaeoacremonium* species have been identified on the basis of morphological traits and DNA phylogeny, which have proven to be pathogenic to young vine plants, causing brown wood streaking symptoms (Mostert *et al*., 2006). At present, the two main mitosporic fungi associated with esca are *Phaeoacremonium aleophilum* and *Phaeomoniella chlamydospora* (Larignon and Dubos, 1997; Mugnai *et al*., 1997). They are reported to be casual agents of Petri disease in young plants, although they are also involved in esca disease in older grapevines (Mugnai *et al*., 1999; Sparapano *et al*., 2000).

Recent studies combining morphological methods and molecular techniques (Cortesi *et al*., 2000; Fischer, 2000) have demonstrated that what was identified for a long time as *Phellinus igniarius* actually corresponds to *Fomitiporia punctata* (=*Phellinus punctatus*), the most common hymenochaetaceous basidiomycete associated with white rot of vines in European vineyards, especially in Italy, France and Spain. Therefore, it is commonly accepted that previous records of *P. igniarius* classified on the basis of *in vitro* cultures probably correspond to *Phellinus* (*Fomitiporia*) *punctatus*.

However, the systematic status of this lignicolous basidiomycete has recently been redefined. A new taxon, *Fomitiporia mediterranea* M. Fischer has been described (Fischer, 2000), providing a new taxonomic status to a collection of isolates usually identified as *F. punctata*, sampled from vineyards in Italy and Germany. *Fomitiporia australiensis*, another new taxon associated with white heart rot of esca-diseased grapevines in Australia has also been described recently (Fischer *et al*., 2005). In both cases, macro and microscopic traits of the basidiocarps of these new taxa are very similar to those produced by *F. punctata.*

Other genera involved in grapevine trunk diseases have been reported. Several species of *Botryosphaeria*-like fungi associated with grapevines have been identified, employing both morphological (Tuset *et al*., 1980; Tuset and Portilla, 1987) and molecular analyses (Slippers *et al*., 2004; Úrbez-Torres *et al*., 2006, Slippers *et al*., 2007) in America and several European countries, including Spain. Some species of the genus *Phomopsis* (Coelomycetes, mitosporic fungi) have traditionally been associated with esca symdrome (Tuset, 1977; Tuset and García, 1977). *Phomopsis viticola* Sacc., the causal agent of "American excoriose" (Pearson and Goheen, 1994) together with *Phomopsis vitimegaspora*, have also been confirmed as pathogens of grapevines (Kuo and Leu, 1998; Niekerk *et al*., 2005).

Etiology of esca under the specific cultural methods and Mediterranean conditions in Spain remains unclear. In order to design rational disease control strategies it is essential to know the occurrence of the causal agents in a specific environment. The aim of this work was to identify and characterize the causal agents of esca using both morphological methods and rDNA molecular analyses. Moreover, the association of these fungal pathogens with vine parts will provide greater insight into population dynamics of esca disease.

Material and methods

Plant material

During 2003-2005 samplings were carried out in eight experimental plots in vineyards throughout the Comunidad Valenciana (Eastern Spain), covering all the different bioclimatic variants in this region (Table 1). The vineyards studied comprised eight varieties of grapevines, namely Bobal, Garnacha, Giró, Italia, Merlot, Monastrell, Moscatel and Tempranillo. Sampling

Table 1. Localities of vineyards and cultivars surveyed along the Comunidad Valenciana during 2003-2005. Experimental plots (surface 100 m^2), are located by UTM coordinates

Province	Locality	UTM	Vine cultivar	Survey year
Alicante	Llíber	31S 2395 42928	Giró	2003
Alicante	Monforte	30S 6984 42494	Italia	2003, 2004, 2005
Alicante	Moraira	31S 2497 42877	Moscatel	2003, 2005
Alicante	Pinoso	30S 6990 42507	Monastrell	2003, 2004, 2005
Alicante	Teulada	31S 2487 42899	Moscatel	2003, 2004, 2005
Valencia	Chiva	30S 6871 43712	Merlot	2003, 2005
Valencia	El Rebollar	30S 6693 43715	Bobal, Garnacha, Monastrel, Tempranillo	2003, 2004, 2005
Valencia	Quatretonda	30S 7256 43133	Monastrel, Tempranillo	2003, 2004, 2005

plots were located in the vineyards themselves or nearby, comprising adult vines of over 20 years old that showed characteristic esca symptoms in both severe (the so-called apoplexy) or mild forms. Young vines (1 to 4 years old) from vineyards and mother plants from randomly selected vine nurseries were also sampled. Sampling was carried out randomly three times per year (spring, autumn and winter) in the three-year period assayed. Approximately 600 plants or plant fragments showing esca symptoms were sampled, identified, labeled and stored at 10ºC in the dark for further processing.

Isolation of fungi

To isolate the fungi associated with esca and achieve better correlation with vine part occurrence, the sampled vines were divided into three parts: trunk, canes and young vine shoots. The surface of the respective parts was visually examined for presence of basidiocarps or any other symptoms. Then, different vine parts were peeled and sectioned off longitudinally and the surface sterilized twice with 2% sodium hypochlorite for 20 min. Once samples were dry, a sterile scalpel was used to slice 20 wood chips ($\cong 5 \times 5 \times 1$ mm) from inner diseased wood and from the surface as well as from (apparently) healthy trunk and cane wood. Similarly, chips from young vine shoots were obtained separately from pith and cortex. Sterile forceps were used to place all the pieces of wood in 9 cm Petri dishes containing potato dextrose agar (PDA) medium supplemented with 100 g mL^{-1} of streptomycin to avoid bacterial contaminants. Emerging fungal colonies were further subcultured to obtain pure cultures either on PDA or malt agar (MA) medium. Petri dishes containing wood chips were incubated at 23ºC until species identification.

Morphological identification

Identification and morphometrical tasks were carried out by direct observation using an optical microscope (Leica DML52) with an incorporated image-capturing device. Microscope slides of the fungal material (either cultured mycelia or fruitbody structures) were examined under binocular lens Leica (Wild Mod. M3C) with an incorporated cold-light device. Distilled water or lactophenol-cotton blue was used as mounting media, depending on the observation. Teleomorphic and anamorphic identifications were performed by observing and measuring several somatic and reproductive structures using UTHSCSA Image Tool software. Once all the observable features and measurements for each isolate were recorded, determinations were made consulting the taxonomic literature available for each of the fungal groups (Barnett, 1955; Cunningham, 1965; Stalpers, 1978; Larsen and Cobb-Poulle, 1992; Ryvarden and Gilbertson, 1994; Crous *et al*., 1996; Fischer, 2000, 2002; Niekerk *et al*., 2005).

Statistical analysis

Frequency data were expressed as the average of frequency observed in three replicas with their respective standard errors. Frequency represents the number of plant showing a particular fungus per total plants studied. To compare the presence of each fungus among all vine parts studied a chi-squared test was performed.

Molecular identification

DNA extraction

Mitosporic fungi were grown on PDA plates for at least 7 days at 23°C. YPD liquid medium inoculated with a spore suspension of each pure culture was grown for 48 h at 23ºC. Mycelium was filtered, lyophilized and stored at room temperature. Basidiomycetes DNA isolation was performed using fresh mycelia tissue. DNA isolation was then performed as previously described by Le Cam *et al*. (2002) with minor modifications. The eluted DNA was stored at –20°C in Tris-EDTA buffer and used as template for PCR reactions.

DNAs were subjected to PCR reactions with primers ITS1 and ITS4 (White *et al*., 1990). ITS1-ITS4 is a pair of universal primers widely used to identify fungi. This primer pair detects the species variability in the internal transcribed spacers and 5.8S rRNA gene region (600-650 bp), and are located in the 18S and 28S flanking regions.

PCR amplification

PCR reactions were performed in a total volume of 100 µL containing 1 µL (20 to 60 ng) of template DNA, 1 µM each primer, 200 µM each dNTP, and 1.25 U of *Taq* DNA polymerase (Invitrogen, MD). Cycling parameters were 94°C for 5 min followed by 30 cycles of 94°C for 30 s, 52°C for 45 s, and 72°C for 1 min. Amplification products were analysed by electrophoresis through 1.5% agarose in TAE buffer.

DNA sequencing

PCR products were purified using the Ultra Clean TM PCR Clean-up (MoBio Laboratories Inc., California) and then sequenced using primers ITS1 and ITS4 on both strands. DNA sequencing was performed using the fluorescent chain-terminating dideoxynucleotides method (Prober *et al*., 1987) and an ABI 377 sequencer (Applied Biosystems, Madrid, Spain). Nucleotide sequence data of ITS region were compared with all sequences present in GenBank database using the Washington University-Basic Local Alignment Search Tool (WU-BLAST) algorithm (Altschul and Gish, 1996). When appropriate, sequences were aligned using the ClustalX (v 1.64b) program (Thompson *et al*., 1997).

Results

Fungal isolation and morphological identification

Fungi were isolated from different tissues of esca-diseased grapevines sampled in this study, followed up by morphological characterization. Isolates were characterized according to different aspects, such as conidiophoros, pycnida, size or shape of spores (Table 2) and the following fungi were identified: *Diplodia mutila* Shoemaker *(Dmu)*, *Phaeoacremonium aleophilum* W. Gams, Crous, M.J. Wingf. and Mugnai (*Pal*), *Phaeomoniella chlamydospora* (W. Gams, Crous, M.J. Wingf. and L. Mugnai) Crous and W. Gams (*Pch)* and *Phomopsis viticola* (Sacc.) Sacc. (*Pvi*) and the basidiomycetes *Fomitiporia mediterranea* M. Fisch. (*Fm*) and *Stereum hirsutum* (Willd.) Pers. *(Shi)*. Additional fungi isolated from vine tissues included *Alternaria alternata* (Fr.) Keissll., *Aspergillus niger* Tiegh, *Botrytis cinerea* Person., *Chaetomium globosum* Kunze, *Cladosporium* spp., *Coniothecium* spp., *Cytospora* spp., *Diplodina* spp., *Fusarium* spp., *Penicillium* spp., *Phoma* spp., *Seimatosporium lichenicola* (Corda) Shoemaker and E. Müll *Trichoderma aureoviride* Rifai and *Truncatella* spp. Of these, only *Trichoderma aureoviride* was further taken into account because its presence may predispose vine tissue to colonization by esca fungi. The rest of the isolated fungi were not considered further in this study since they occur as facultative parasites on any decayed wood.

All the symptoms usually associated with and/or described for the several esca-related fungi were continually observed during field samplings. These included typical foliar symptoms (Fig. 1a), sudden wilting and death of entire plant due to aploplexy (Fig. 1b), plants showing cortical Phomopsis cane and leaf spot frequently caused by *Pvi* strains (Fig. 1c) and diseased bark patches where typical *Dmu* pycnidia were observed (Fig. 1d). Moreover inner wood was found exhibiting a typical black halo surrounding decayed, wet-rotted areas from which both *Pal* and *Pch* strains were isolated (Fig. 1e-f). Other symptoms were spongy, white-rotted wood, from which *Fm* strains were mostly isolated, just in the same way as *Shi* (Fig. 1g-h).

Frequency of fungi associated with esca

Figure 2A shows the frequency analysis of esca fungi isolated in surveys carried out from 2003 to 2005. Sur-

Table 2. Morphologic characteristics and molecular characterization of esca associated fungi in vines surveyed

Isolated fungi	Morphologic characters		Sequencing			
	Fruitbodies	Spores	Accession number[a]	Size (bp)[b]	Accession number[c]	% of Identity[d]
Diplodia mutila Shoemaker	Pycnidia	Conidia smooth, unicellular, mostly hyaline and aseptate	EU856764	578	AY259093	99%
			EU856765	578		100%
			EU856766	578		100%
Phaeoacremonium aleophilum W. Gams, Crous, M.J. Wingf. and L. Mugnai	Monophialidic conidiogenopus cells	Conidia oblong-ellipsoid to cylindrical, sometimes curvate	EU851104	614	AY644479	100%
			EU851105	617		99%
			EU851106	614		99%
Phaeomoniella chlamydospora (W. Gams, Crous, M.J. Wingf. and L. Mugnai) Crous and W. Gams	Macronematous conidiophores	Conidia subhyaline, oblong-ellipsoid to ovoid, straight	EU851101	524	EU018416	100%
			EU851102	525		99%
			EU851103	524		99%
Phomopsis viticola (Sacc.) Sacc.	Pycnidia	Ovate to fusoid alpha-conidia, tohether with filiform with acute ends, curvulate beta-conidia	EU851107	569	AY662404	98%
			EU851108	569		98%
			EU851109	568		98%
Fomitiporia mediterranea M. Fisch.	Perennial, resupinate to effuse with porate hymenial surface	Smooth, hyaline, ovoid to globose basidiospores	EU851115	752	AY529688	94%
			EU851116	750	AY529688	94%
			EU851117	749	AY529688	94%
			EU851118	740	EU477479	100%
			EU851119	755	AY780427	96%
Stereum hirsutum (Willd.) Pers.	Annual, pileate to effuse-reflected with smooth hymenial surface	Smooth, hyaline, elliptic to cylindrical basidiospores	EU851110	615	AY854063	99%
			EU851111	618		98%
			EU851112	590		99%
			EU851113	615		99%
			EU851114	590		99%

[a]: Accesion numbers of PCR products purified and sequenced. PCR was performed with primers ITS1 and ITS4, using DNA of three or five isolates of each fungus. [b]: Excluding primer sequences. [c]: Accesion number of the sequence with highest similarity that allow fungal identification. [d]: Highest identity determined for sequence comparison using WU-BLAST algorithm against EMBL.

veyed vines in 2003 showed *Dmu* (70%), *Fm* (45%), *Shi* (42%) and *Pch* (37%) as the most frequently isolated fungi. In contrast, *Pal* (20%) and *Pvi* (10%) presence was less frequent in this year, occurring only occasionally in vines with simultaneous symptoms of esca and excoriosis. In 2004 *Dmu* was found in 100% of vines surveyed, followed by *Pal* and *Pch* (both 75%). *Fm* (65%), *Pvi* (50%) and *Shi* (50%) also exhibited high frequency from the several samplings carried out during that year. Finally, in the vines surveyed in 2005, both *Shi* (70%) and *Fm* (65%) were the most commonly isolated fungi followed by *Dmu* (40%), *Pch* (23%), *Pal* (18%) and *Pvi* (10%).

The different fungal strains associated to esca symptoms were also analyzed with respect to grapevine varieties (Fig. 2B). In some cases varieties appeared to be restricted to the exclusive presence of *Dmu* as observed in Bobal and Merlot. In fact, *Dmu* was the only fungus that was present in all varieties surveyed, exhibiting the highest frequency in all of them with the only exception being Garnacha and Giró, in which *Pch* was the most abundant fungus. By contrast, *Pvi* was the least present fungi, observed only in Tempranillo. In Italia, *Dmu* and both basiodiomycetes, *Fm* and *Shi* were the only strains present. In the rest of varieties (Garnacha, Giró, Monastrell and Moscatel) all fungal strains (with the exception of *Pvi*) were observed, with higher incidence of *Pch* compared to *Pal*, and also higher presence of *Fm* compared to *Shi*. Tempranillo was the only variety in which all fungal strains were observed.

All esca-associated fungal strains were also examined concerning the different localities surveyed (Fig. 2C). *Dmu* was present in all plots surveyed, *Pal* and *Pch* were absent in Monforte and Chiva, and *Pvi* with 34% frequency, was only found in Quatretonda. Both basidiomycetes *Fm* and *Shi* were not present in Monforte, Moraira and Pinoso and *Shi* was also absent in El Rebo-

Figure 1. Symptoms associated with the several esca-related fungi. a) Typical foliar symptoms. b) Sudden wilting and death of entire vine due to apoplexy. c) Cortical 'Excoriosis' frequently caused by *Phomopsis viticola.* d) Typical *Diplodia mutila* pycnidia. e and f) Inner wood exhibiting a typical black halo surrounding decayed wet-rotted areas caused by *Phaeoacremonium aleophilum* and *Phaeomoniella chlamydospora* respectively. g and h) Spongy, white-rotted wood caused by *Fomitiporia mediterranea* and *Stereum hirsutum* respectively.

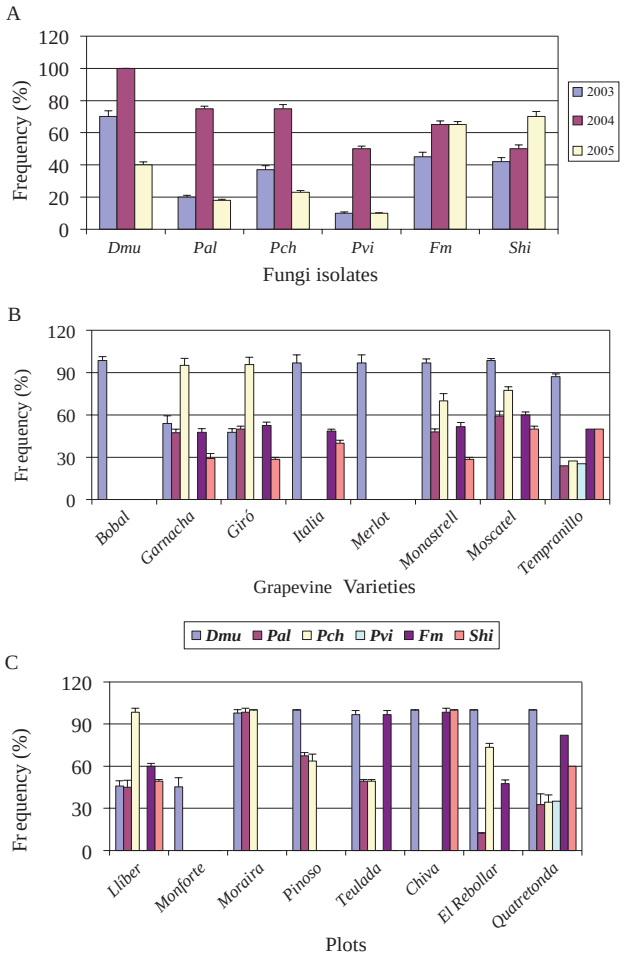

Figure 2. Frequency of esca-associated fungi regarding to different years surveyed (A), grapevine varieties (B) and in the different plots surveyed (C). Frequency is expressed as number of plants showing a particular fungus per total vine plants studied. Values were calculated as the average of frequency observed in three replicas. Number of plants assayed varies in each case. Standard errors of the mean are indicated by bars. *Dmu = D. mutila, Pal= Phaeo. aleophilum, Pch= P. chlamydospora, Pvi= Pho. viticola, Fm= F. mediterranea Shi= S. hirsutum.*

llar and Teulada. Quatretonda was the only surveyed region in which all strains were found. The incidence varied throughout all plots sampled and *Dmu* was present at high levels (97-100%) in all regions with the exception of Llíber and Monforte. *Pal* exhibited the highest incidence in Moraira (98%) followed by Pinoso, Teulada, Llíber, Quatretonda and El Rebollar. *Pch* was recorded at a high frequency, ranging from 100-64%, in Moraira, Llíber, El Rebollar and Pinoso and values of 49% and 34% in Teulada and Quatretonda, respectively. Where both basidiomycetes were present *Fm* was registered at almost the same or higher incidence as compared to *Shi*.

Association of esca fungi with vine parts

Trunks, canes and young vine shoots were studied separately for occurrence and incidence of esca-associated fungi. Considering all the three vine parts studied, *Dmu* was clearly the most frequently isolated fungus in our analysis (Fig. 3) being present in all

parts studied. Regarding each vine part separately, certain differences were found. Trunk isolations mainly comprised *Dmu* (58%), *Shi* (56%) and *Fm* (50%), this latter was isolated from spongy and decayed yellowish wood, never from healthy wood, and *Pch* (32%) was mainly isolated from a black halo surrounding the decayed wood and also from healthy-looking wood. *Pal* (12%) was found to be less common in the trunk and not clearly related to any wood symptom and *Pvi* was not observed. In canes, all fungal strains were observed: *Dmu* (82%), followed by *Shi* (27%), *Fm* (25%) and *Pal* (21%). However, the presence of both basidiomycetes was less abundant and *Pal* increased its relative presence compared to what it was observed in trunks. *Pvi* and *Pch* were less frequent in cane (11% and 5%, respectively). Symptoms observed in cane sections were very similar to those in trunks, with the exception of yellowish spongy, white-rotted areas, which were never observed in canes. In vine shoots, *Dmu* (28%) and *Pvi* (16%) were the only isolated fungi. *Dmu* was mostly isolated from diseased bark patches showing a continuous black spot advancing from the tip, while *Pvi* was obtained from typical excoriosis spots on the bark. A statistical analysis using the chi-squared test was performed to check whether the fungal distribution was similar in the different vine parts. Data obtained (Table 3) confirmed that *Dmu* was present at significantly higher rates in canes than in trunks or shoots; all basidiomycetes as well as *Pch* were present at a significantly higher rate in trunks than in canes; there were no significant differences between trunks and canes for *Pal* presence; nor was there a significant difference between canes and shoots for *Pvi* presence.

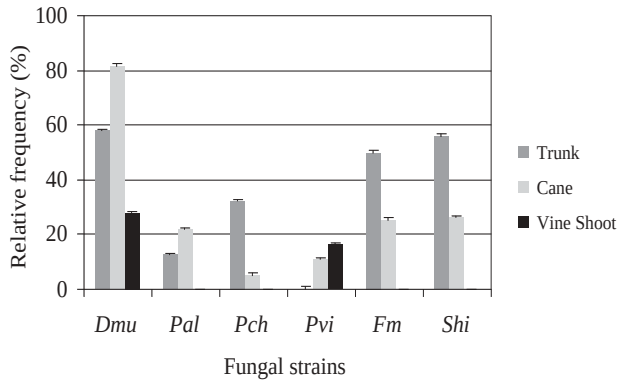

Figure 3. Incidence of esca fungi in vine parts: percentages of number of vine parts showing a particular esca-associated fungus isolated per total vine parts sampled. Standard errors of the mean are indicated by bars. *Dmu = D. mutila, Pal= Phaeo. aleophilum, Pch= P. chlamydospora, Pvi= Pho. viticola, Fm= F. mediterranea Shi= S. hirsutum.*

Molecular characterization

Comparisons of nucleotide sequences of five different isolates of both basidiomycetes (*Fm* and *Shi*) and three of each mitosporic fungus (*Dmu, Pal, Pch and Pvi*) with all sequences present on available databases confirmed most of the previous taxonomic identifications with some minor exceptions (Table 2). All sequences from the different isolates of *Dmu, Pal, Pch, Pvi* and *Shi* had 98-100% of homology with the sequences of other isolates present in databases. The several *Pal* isolates sequences obtained in this work had 100% homology with sequences of *Togninia minima* (Tul. & C. Tul.) Berl. (Calosphaeriales, Ascomycotina) teleomorph of this fungus. Nevertheless, *Togninia minima* (the perfect state) was not found in any of the infected plants surveyed.

Most of the hymenochaetaceous basidiomycetes isolated were morphologically identified either as *F. punctata* or *Fm*. The rDNA sequence analysis of five isolates, together with a more in-depth morphological study of *in vitro* cultures and fruitbodies, demonstrated that all these isolates could be assigned to *Fm* (database sequences AY529688, EU477479 and AY780427). Nucleotide divergence of the ITS region observed among *Fm* sequences obtained here, and other sequences from GenBank representing *F. punctata*, were 7.2% on average. In fact, among all *Fm* sequences identified in the Comunidad Valenciana, some of them (EU851115, EU851116 and EU851117) presented 94% of homology with another *Fm* sequence isolated from grapevines (AY529688) while the other two (EU851118 and EU85119) had 100% and 96% of homology with EU477479 and AY780427, obtained from hazelnut orchards and *Platanus x acerifolia*, respectively.

Concerning *Botryosphaeria*-like fungi, after the integration of both morphological and molecular data, most of the isolates sequenced represented *Dmu* (*Botryosphaeria stevensii)* with one exception (EU851098, under additional study), that could represent *Botryosphaeria rhodina* (*Lasiodiplodia theobromae*), on the basis of its nucleotide sequence and conidial features.

Table 3. Statistical analysis performed using chi-squared test to monitor fungal strains distribution in all vine parts studied (trunk, cane and vine shoot). Data correspond to p-values

Fungal strain	Trunk *vs* Cane	Trunk *vs* Shoot	Cane *vs* Shoot
Diplodia mutila	0.0043	0.0008	<0.0001
Phaeoacremonium aleophilum	0.1460	—	—
Phaeomoniella chlamydospora	<0.0001	—	—
Phomopsis viticola	—	—	0.4225
Fomitiporia mediterranea	0.0040	—	—
Stereum hirsutum	0.0010	—	—

Discussion

Esca decline has been termed an 'elusive' disease not only because of the diversity and range of its symptoms, but also because the pathogens involved in the appearance and development of esca still remain unclear (Mugnai *et al.*, 1999). Previous reports have pointed out the existence of two main groups of fungi involved in esca symptoms and probably acting in ecological succession (Graniti *et al*., 2000). Although several attempts have been made to reproduce the white rot lesions and/or external symptoms (Péros *et al*., 2008) Koch´s postulates have not been totally fulfilled (Cortesi *et al*., 2000; Feliciano *et al*., 2004).

The wood decay symptoms of esca have been associated to several fungi and many authors have described the presence of *Phaeoacremonium inflatipes, Pal* and *Pch* associated to young grapevine decline (Whiting *et al*., 2001). However, in our study we were only able to isolate strains of *Pal* and *Pch* as part of the mitosporic fungi associated to esca disease. *Pch* was found to coexist simultaneously with lignicolous basidiomycetes *Shi* and *Fm*, which appeared in old vines infected for a long time. Interestingly, most isolations of *Pch* were made from trunks, either alone or combined with *Shi* and chi-square testing did indicate significant presence in trunks compared to canes. However, *Pch* was infrequently isolated from canes, questioning its ability to enter the vines through cane pruning wounds, as reported by Larignon and Dubos (2000).

Besides, other fungi such as *Pvi* and *Dmu* (and *B. rhodina* to a lesser extent) have also been isolated from Comunidad Valenciana grapevines showing esca symptoms. *Pvi* isolated either from canes or young vine shoots of a few esca-affected vines did not display any significant differences, suggesting that esca and excoriosis could occur simultaneously in the same vine stands. Among the numerous *Botryosphaeria* species associated with diseased grapevines, *Dmu* was repeatedly isolated in all the plant material analyzed, with the exception of one single strain, possibly co-specific with *B. rhodina*, which is the most frequently reported *Botryosphaeria* in Californian grapevines (Úrbez-Torres *et al*., 2006). Different *Botryosphaeria* species are reported to be associated with a broad range of grapevine symptoms (Úrbez-Torres *et al*., 2006). *Dmu* was associated with either wedge or half-moon shaped lesions of grapevines or sectorial vascular necrosis (Úrbez-Torres *et al*., 2006). Although single attack does not lead to full esca symptoms, droughts affecting vineyards could enhance its pathogenic potential, indirectly increasing vine susceptibility to other esca-related fungi. In contrast with our findings, previous studies of esca incidence in some vine areas in Spain (Armengol *et al.*, 2001) reported that *B. obtusa* was the most commonly isolated species of this genus from diseased grapevines. Some authors have shown that the presence of different *Botryosphaeria* spp. is related to grapevine-cultivar susceptibility (Úrbez-Torres *et al*., 2006). However, our results indicated that *Dmu* was present in all grapevine varieties subject of this study. This is a clear example of controversy concerning pathogenicity of *Botryosphaeria* and hence, factors involving the presence of a particular taxon remain unknown.

In our work, *Shi* and *Fm* were the second most predominant species isolated, confirming the results obtained in Italy (Mugnai *et al.*, 1999) and in Spain (Armengol *et al.*, 2001), under similar Mediterranean climate conditions. This would suggest that both species are highly adapted to dry conditions and that their isolation frequency could depend on annual temperature and humidity. Despite this, our findings do not lead to the conclusion that climatic conditions are the main reason for basidiomycete's predominance, as during the three-year survey there was only a slight difference in rainfall in 2005. We believe that the length of time needed by these

ligniculous basidiomycetes to colonize and decay the woody tissues of vines might explain why they were found predominantly in old trunks (and to a lesser extent in canes) during the last two years of sampling.

Among the numerous fungi causing esca, *Shi* is usually mentioned in the literature as a minor component. Mugnai *et al*. (1999) pointed out that in Italy white rot of vine wood was only very infrequently associated with *Shi*, although this species readily forms fruitbodies on the surface of plant hosts. This, together with our own field investigations, where basidiocarps of *Shi* were frequently observed in most vineyards surveyed, suggests that this fungus could act mostly as a weak facultative parasite, confined to the external layer of the wood. Occasionally *Shi* can penetrate (via small wounds such as pruning cuts, etc) the inner layer of the wood and produce a very limited infection and decay in the colonized plants as observed in canes.

Fm is described as a new wood-decaying basidiomycete species associated with esca of grapevine in European wine-growing countries (Fischer, 2002). It is now commonly accepted that strains of *Fomitiporia* isolated from diseased grapevines in southern European countries, must be assigned to *Fm*, a taxon recently separated from the *F. punctata* complex (Fischer, 2002). Interestingly, *Fm* is reported to be restricted to vineyards in central and northern Europe, whereas in the southern European countries this taxon not only occurs in *Vitis*, but in several other hardwood genera, just like *F. punctata*, due to its broad ecological range (Cunningham, 1965; Larsen and Cobb-Poulle, 1992; Ryvarden and Gilbertson, 1994). Several authors (Cortesi *et al*., 2000; Fischer, 2000, 2002) suggest that this fact could play an important role in esca disease epidemics, since host plants situated in the vicinity could act as a source of potential inoculum for *Fomitiporia* strains. However, we did not find basidiocarps of both *Fm* and *F. punctata* parasitizing cultivated trees such as *Ceratonia siliqua*, *Olea europaea*, *Prunus spp*. etc., located near the numerous vineyards sampled. Morphological distinction among basidiocarps or cultures of these taxa is usually hindered by the absence of sufficient diagnostic features; therefore the use and comparison of genomic data could discriminate them more easily at species level. In order to perform a complete characterization of *Fomitiporia* isolates, preliminary phylogenetic analyses were carried out based on the comparison of rDNA (18S and 28S partial sequence and ITS1, 5.8S, and ITS 2, complete sequence) data of these strains and sequences representing isolates of *Fm*, *F. punctata* and other related *Phellinus* species obtained from available databases. These analyses indicate that all strains isolated during the period 2003-2005 could be assigned to *Fm*. All esca-related *Fomitiporia* species have been recognized as a complex, thus ecological, microbiological and molecular data may facilitate recognition of new species within this group of hymenochaetaceous white-rot fungi associated with esca syndrome.

The sporadic expression of esca symptoms is reported to be due to climatic parameters such as rainfall and temperature (Redondo *et al*., 2001; Surico *et al*., 2006). But as mentioned above, our data do not support this and we believe that there are many conditions affecting esca disease (symptoms and fungal strains presence) and spread of esca, such as grapevine age, crop management (Fussler *et al*., 2008) and in some cases, different cultivars and growing regions, since many differences can be observed on comparing esca disease in France with Italy or Spain. Our results indicate that not all grapevine varieties were susceptible to the different fungal strains found in this study and that are associated to esca. In fact, *Pvi* was restricted to Tempranillo and *Dmu* was the only one present in all grapevines, being the only fungus in Bobal and Merlot. Greater differences were found in growing regions represented as plot frequency. The presence of the different fungal strains was variable but again *Dmu* was the only one present in all plots surveyed and Quatretonda was the only region where all fungal strains were present. Nevertheless, the role played by variety and growing region, within the Mediterranean, in fungal strain incidence remains unclear since other authors have reported the presence of other fungi in the same grapevine varieties examined in this work (Armengol *et al*., 2001; Úrbez-Torres *et al*., 2006). The incidence of fungal strains could be more closely correlated to their ocurrence depending of the season or year under survey, grapevine age and/or crop management than on the grapevine variety or growing region.

All taxonomic data corresponding to esca symptoms obtained over a number of years as results of morphological identification and growth were validated and confirmed employing molecular tools. Results of this study have provided a record of the occurrence of some of the numerous fungi associated with esca disease affecting vineyards in Spain, and particularly the significance of fungal strains' distribution in the different vine parts surveyed. Furthermore, the current study provides evidence of the identity and systematic placement of taxa involved in grapevine wood decay. Nevertheless, more

accurate and extensive molecular studies are required in order to provide a reliable phylogenetic recognition of new taxa associated to esca syndrome in southern European vineyards and sensitive methods must be developed to follow up esca disease distribution.

Acknowledgements

This work was financed by the Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA) of Spain under projects VINO1-034-C2-1 and RTA2005-00042-C02-01 and FEDER funds. We acknowledge Concha Hinarejos and Jose Luis Mira for their excellent technical assistance. We also acknowledge the Biometric Department of IVIA for their valuable help in the statistical analysis.

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