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Fungi associated with esca and grapevine declines in Spain: a three-year survey

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Summary: A survey of 140 vineyards in different production areas of Spain was conducted from 1999 to 2001 to identify pathogenic grapevine wood fungi. Fungal infected vines showed esca and decline symptoms, consisting mainly of reduced growth of canes and shoots, yellowing and necrotic spotting of leaves, sectorial and central brown necrosis of the trunk, soft rotted tissues and discoloured xylem at the base of the vines. Several fungi were consistently found associated with these symptoms: *Botryosphaeria obtusa* (isolated from 61.4% of all vineyards studied), *Phaeoacremonium aleophilum* (26.4%), *Cylindrocarpon* spp. (20%), *Phaeomoniella chlamydospora* (18.6%) and *Fomitiporia punctata* (15%) were the main fungi found. Less frequently isolated species were: *Botryosphaeria dothidea* (6.4%), *Eutypa lata* (2.1%), and *Stereum hirsutum* (1.4%). The association of these fungi and their role in esca disease and grapevine declines, as well as in the decline of young grapevines in Spain are discussed.

Key words: RFLP analysis, *Vitis vinifera*, wood fungi, young grapevines.

Introduction

Esca and grapevine declines have become a limiting factor for many grapevine production areas around the world. It has been suggested that changes in cultural practices such as reduced sanitary care of rootstocks and vine propagation material, poor protection of pruning wounds and the banning of or restrictions in the use of sodium arsenite have contributed greatly to the steady increase of these diseases over the last 10–15 years (Chiarappa, 2000; Graniti *et al*., 2000).

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As a consequence, research in trunk diseases of grapevine and related subjects has also progressed. Several fungi have been found to be associated with esca and other declines. They include, among many others, basidiomycetes such as *Fomitiporia punctata* (Fr.) Murrill, the ascomycetes *Eutypa lata* (Pers.:Fr.) Tul. & C. Tul. and *Botryosphaeria obtusa* (Schwein.) Shoemaker, and mitosporic fungi (hyphomycetes) such as *Phaeoacremonium aleophilum* W. Gams, Crous, M.J. Wingf. & L. Mugnai and *Phaeomoniella chlamydospora* (W. Gams, Crous, M.J. Wingf. & L. Mugnai) Crous & W. Gams (Larignon and Dubos, 1997; Scheck *et al*., 1998; Mugnai *et al*., 1999; Graniti *et al*., 2000).

In recent years the incidence of esca and grapevine declines has also increased in Spain, but although Spain is one of the world's largest wine pro-

ducers, to date no systematic analysis has been carried out to evaluate the occurrence and incidence of the fungi associated with trunk diseases on grapevine. The aim of this work was to study the current state of esca and grapevine decline in Spain, by determining the identity of the main grapevine wood fungi and the percentage of vineyards (out of 140-vineyard sample in various production areas) infected with each of them in a 3-year period (1999–2001).

Materials and methods

Sampling

One hundred and forty vineyards from the main grapevine production areas of Spain were surveyed from 1999 to 2001 to study the fungi associated with trunk diseases. Vineyards of different ages were selected in Andalucía, Aragón, Castilla la Mancha, Castilla y León, Cataluña, Comunidad Valenciana, La Rioja, Murcia and the Navarra regions. The vines that were examined showed esca and decline symptoms consisting mainly of reduced growth of the canes and shoots, yellowing and necrotic spotting of the leaves, sectorial and central brown necrosis of the trunk, soft rotted tissues and discolored xylem at the base.

Fungal isolation from individual vines

Affected vines were collected in variable number from each vineyard (at least 3–5 vines per vineyard) and systematically analyzed to identify the wood fungi they contained. Cross sections of the woody stem of the rootstock and of each vine cultivar were examined. Small pieces of discolored or decayed tissues were plated on malt extract agar (MEA) supplemented with 0.5 mg ml^{-1} of streptomycin sulphate. Cultures were incubated at 25–27°C in the dark. Isolates were transferred to potato dextrose agar (PDA) or MEA plates, incubated at room temperature with a 12-h photoperiod and identified according to their morphological characteristics.

Molecular identification of *Fomitiporia punctata*

The basidiomycete *F. punctata* was identified by analysis of DNA amplified from the nuclear-encoded ribosomal RNA (rRNA) gene cluster and restriction analysis of the amplified products (Cortesi *et al*., 2000). The polymerase chain reaction (PCR) was used to amplify a portion of the rRNA subunit, defined by the primer combination ITS1 and ITS4. For restriction analysis the restriction enzymes *Alu*I and *Csp6*I were used, and the results were analyzed using Kodak Digital Science 1D software (Eastman-Kodak, Rochester, NY, USA).

Results

The number and percentages of vineyards with esca or decline fungi are presented in Table 1. Based on the most common disease encountered, vineyards were divided into either: vineyards with

Table 1. Isolation of fungi from sampled vineyards from 1999 to 2001.

Fungus	Vines with esca symptoms		Vines with decline symptoms		Total	
	No. of vineyards	%[a]	No. of vineyards	%[a]	No. of vineyards	%[a]
Botryosphaeria dothidea	2	3.8	7	8	9	6.4
Botryospheria obtusa	43	81.1	43	49.4	86	61.4
Cylindrocarpon spp.	3	5.7	25	28.7	28	20
Eutypa lata	0	0	3	3.4	3	2.1
Fomitiporia punctata	18	34	3	3.4	21	15
Phaeoacremonium aleophilum	15	28.3	22	25.3	37	26.4
Phaeomoniella chlamydospora	4	7.5	22	25.3	26	18.6
Stereum hirsutum	2	3.8	0	0	2	1.4

[a] Percentages were calculated on 140 vineyards: 53 with vines showing symptoms of esca and 87 with vines showing decline symptoms.

esca symptoms (yellowing and necrotic spotting of leaves, wood necrosis and spongy rot), or vineyards with decline symptoms (reduced growth of canes and shoots, sectorial necrosis and decline of young vines). Many fields from either group contained both vines with esca and vines with decline symptoms.

The most common fungus, found in 61.4% of all vineyards, was *B. obtusa*. *P. aleophilum* and *P. chlamydospora* were isolated from 26.4% and 18.6% of all vineyards respectively. *Cylindrocarpon* spp. was identified in 20% of all vineyards and *F. punctata* in 15%. The other fungal pathogens, *B. dothidea*, *E. lata* and *S. hirsutum*, were less frequent, occurring in only 6.4, 2.1 and 2% of all vineyards respectively.

F. punctata was identified by molecular analysis of mycelial isolates. The universal primers ITS1-ITS4 were used to obtain a PCR product of the expected size (750 bp) for all isolates studied. After digestion with the restriction endonucleases *Alu*I and *Csp6*I, restriction fragments from amplified DNA of isolates showed the pattern indicated by Cortesi *et al*. (2000). For example, for isolates from the Comunidad Valenciana region (Fig. 1), digestion with *Alu*1 generated two fragments of approximately 600 and 215 bp. For *Csp6*I, three fragments of approximately 500, 160 and 135 bp were observed.

Discussion

The main objective of this study was to identify the most prominent grapevine wood fungi in Spain and determine the percentage of vineyards in which they were found.

B. obtusa was the predominant fungal species, being isolated from vines in 61.4% of all vineyards sampled. The frequency of this fungus was high both in vineyards with typical esca symptoms (81.1%) and in vineyards with decline symptoms (49.4%). *B. obtusa* is an ascomycete commonly isolated from the trunks and branches of esca-infected grapevines (Mugnai *et al*., 1999). In this study *B. obtusa* was also detected in association with sectorial wood necrosis similar to that caused by *E. lata*. By contrast, *E. lata* was detected in only 2.1% of sampled vineyards. This finding refutes the common belief that *E. lata* has a high incidence in all vine-growing areas in Spain and that wood necrosis due to this fungus is commonly. In fact *E. lata* is not found at all or is rare in esca-infected vine-

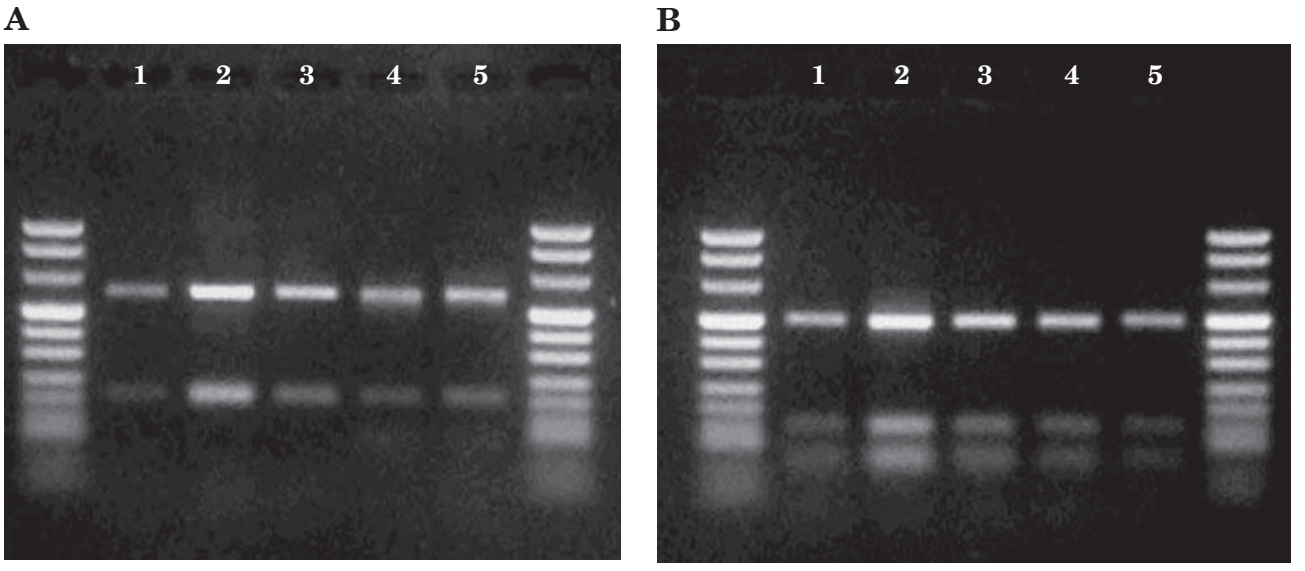

Fig.1. Restriction analysis of internal transcribed spacer portions of the nuclear-encoded ribosomal RNA gene cluster amplified by a polymerase chain reaction, digested with A, *Alu*I or B, *Csp*6I, and electrophoresed in 1.5% agarose gels stained with ethidium bromide. Lanes 1 to 5 contain DNA of *Fomitiporia punctata* isolates obtained from the Comunidad Valenciana region: 1, San Antonio; 2, Benicolet; 3-4 San Antonio; 5, Requena. The unlabelled, outside lanes contain DNA molecular weight marker VIII (Boeheringer GmbH, Mannheim, Germany) as a size standard

yards in vine-growing areas of southern Europe (Mugnai *et al*., 1999).

The importance and incidence of *B. obtusa* in Spain coincides with a recent report of severe damage caused by this pathogen in France (Larignon and Dubos, 2001). These authors state that the disease caused by *B. obtusa* can easily be confused with esca because symptoms of both diseases are similar.

B. dothidea is a related ascomycete that was isolated at low frequency (6.4%). It is associated with small necroses at the base of canes similar to those described by Phillips (1998) in Portugal.

The wood-rotting basidiomycetes *F. punctata* and *S. hirsutum* were isolated from vines in 15% and 1.4% of vineyards respectively. These pathogens were isolated mainly from vineyards with esca symptoms, but *F. punctata* was also isolated from 3.4% of vineyards with decline symptoms. *F. punctata* was clearly associated with wood deterioration such as pale yellow soft rot and spongy decay. Its identification was based on RFLP analysis of mycelium DNA and confirms the occurrence of this fungus also in Spain, where it is commonly misidentified (Fischer, 1996) as *Phellinus igniarius* (Arias, 1992). *S. hirsutum* was found only rarely in the vineyards (1.4%). A low incidence of *S. hirsutum* was reported by Larignon and Dubos (1997) in a study of 309 diseased vines in France, and by Mugnai *et al*. (1996) in Italy.

The hyphomycetes *P. aleophilum* and *P. chlamydospora* were each isolated at similar frequencies betweeen esca-affected vineyards and vineyards with decline symptoms, especially those planted with young vines. Decline symptoms in young grapevines have increased in Spain over the last 3–4 years in areas with new vineyards. Decline-affected vines in this study showed stunted growth and small, discolored leaves. There was darkening of the wood tissue of the xylem vessels in the rootstock. This darkening showed up as spots in cross section and as streaks in longitudinal section. Moreover, a dark-brown gummy substance was observed in the vessels. These symptoms were identical to those in young vines with decline associated with *Phaeoacremonium* spp. and *P. chlamydospora* in different countries (Scheck *et al*., 1998; Mugnai *et al*., 1999; Morton, 2000; Sidoti *et al*., 2000). In some vineyards, *Cylindrocarpon* spp. were also isolated from young vines and from vines with basal rot or root necrosis of the rootstock. *Cylindrocarpon* spp. have also been associated with young grapevine decline (Scheck *et al*., 1998; Rego *et al*., 2000) and have been reported to be the causal agent of black-foot disease in California (Scheck *et al*., 1998) and France (Dumot *et al*., 1999).

In conclusion, the study showed that the fungi causing esca and grapevine decline are the same in Spain as they are in France and Italy, and that the frequency with which they are isolated from infected wood is also similar. It seems that in all these countries different syndromes are produced depending on the incidence of associated fungi, and other factors such as vineyard age and site conditions.

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