Characterization of the Fungi Involved with Grapevine Trunk Diseases in Castilla-La Mancha Region, Spain

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Abstract: Grapevine trunk diseases (GTDs) are currently one of the most important problems for grapevine plant and wine industry, causing important economic cost in Castilla-La Mancha, Spain. Traditionally, GTDs were associated with diseases present in mature grapevines, however in recent years the incidence of young vine decline has been increasing. In this work, from 2009 to 2016, 250 young grapevine plants have been analysed by fungal isolation and characterization. In addition, during 2017, 32 mature plants showing decline symptoms were analysed. Both kinds of plants were analysed in order to identify the main fungal trunk pathogens present in Castilla-La Mancha region. As a result, it was possible to isolate species associated with black-foot disease (Cylindrocarpon-like anamorphs), esca and Petri disease (Phaeomoniella chlamydospora and Phaeoacremonium spp.), and Botryosphaeria dieback. All these pathogens were isolated from young and mature plants, this fact could suggest a common origin. The lack of effective control measures for these diseases makes it necessary to implement an integrated disease management strategy that provides an efficient approach to reduce infections in nurseries and get better results in infected vineyards.

Key words: Grapevine trunk diseases, esca, black-foot, Petri disease, Botryosphaeria dieback.

1. Introduction

Grapevine trunk diseases (GTDs) caused by fungal pathogens are currently considered one of the most important problems threatening vineyards and wine industry in Castilla-La Mancha, Spain. The worldwide economic cost for the replacement of dead grapevine plants has been estimated more than 1.5 billion of dollars per year [1]. However, that cost may be underestimated in view of the individual regional data found in literature, for example nearly 13% of French vineyards are affected by GTDs compared to 22% in Chile [2, 3].

Spain is one of the major vine producers worldwide, with currently over 992,961 ha planted, from which 478,162 of them are located in the Castilla-La Mancha region (Central-Eastern Spain), representing 49.5% of the total Spanish vineyard area and 13% in European Union and almost 6% of the world [4].

GTDs include several diseases which are caused by a wide range of different fungi [1, 5]. These pathogens invade plants mainly through pruning wounds and colonise woody parts of the plants causing a slow decline and, in a number of cases, leading to the vine death. Several species can also penetrate through the root system [6, 7].

Traditionally, GTDs were associated with diseases present in mature grapevines, over 10-year-old, such as esca, Botryosphaeria dieback and eutypiosis [8].

Esca is the most widespread and destructive among these diseases. It can manifest in two ways: the chronic esca and the acute syndrome, also known as apoplexy [9]. For the chronic way, external symptoms consist in light green to chlorotic turning into necrotic spots between the veins and/or along the
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leaf margins [10]. Esca symptoms and their expression over time are highly variable [11]. A plant may express foliar symptoms over a few years, consecutively or not, but will then generally die from apoplexy [1]. The most common wood symptoms are dark brown streaks, visible as spots in cross section, observed in the xylem vessels [8]. Phaeomoniella chlamydospora, Phaeoacremonium minimum and Fomitiporia mediterranea are the main fungi associated with esca disease [12].

Botryosphaeria dieback is caused by several members of the family Botryosphaeriaceae. These pathogens mainly attack the perennial organs of grapevine, causing wood discoloration. Foliar symptoms often are difficult to distinguish from symptoms caused by other fungal pathogens [13].

Eutypa dieback is caused by Eutypa lata. This fungus infects exposed vascular vessels by pruning wounds and symptoms usually appear several years after infection. Plants exhibit small and chlorotic leaves with marginal necrosis. The most common wood symptom is a brown, wedge-shaped necrosis in the cordons [14].

Since the early 1990s an increase in the incidence of these diseases in young plants has been reported worldwide [15]. Petri disease and black-foot disease are the main GTDs associated with young vine decline in new plantations [16]. Moreover, several species of the family Botryosphaeriaceae have also been frequently isolated from declining young plants in grapevine producing regions of the world [15].

Petri disease is closely related with esca because P. chlamydospora and Phaeocremonium spp. are present in vines affected by both diseases. These fungi colonize xylem vessels, causing decline symptoms such as reduced vigour, stunted growth, shortened internodes, reduced foliage, interveinal chlorosis, leaf necrosis and dieback [17].

External symptoms of black foot disease are similar to those of Petri disease [18]. The most characteristic symptom of black-foot disease is the presence of extensive necrosis of wood tissues developing from the base of the rootstock [7]. Originally, Cylindrocarpon spp. are indicated as causal agents of this disease. Currently, several species including the genera Campylocarpon, Cylindrocladiella, Dactylonectria, Ilyonectria and Thelonectria are reported as causal agents of black-foot disease [19].

Presently, there are no available treatments for GTDs control, so prevention and early detection are the recommended management strategies [20].

The aim of the present work was to provide updated knowledge of the GTDs pathogens associated with decay vineyards in Castilla-La Mancha region.

2. Materials and Methods

2.1 Samples

2.1.1 Young Plants
From 2009 to 2016, 250 grapevines from symptomatic young vineyards (< 10 years old) located in Castilla-La Mancha region were collected and analysed. These plants showed decline symptoms such as reduced growth, stunting, shortened internodes and leaf discolorations.

2.1.2 Mature Plants
In 2017, eight adult vineyards showing decay symptoms of different varieties of red (Cabernet Sauvignon, Tempranillo and Bobal) and white cultivars (Airen and Macabeo) were used. Initially, for each vineyard, all vines were classified in four stages of the disease according to their visual symptoms (asymptomatic: 0% of damage; initial: < 25% of damage; medium: 25%-50% of damage; advanced: > 50% of damage). One plant of each stage was collected from each vineyard.

2.2 Methods

2.2.1 Fungal Isolation and Morphological Identification
Segments of three different areas of each plant (graft union, trunk and cordons) were cut
longitudinally and transversely to expose any internal symptom. The segments were sterilized by immersion in 96% alcohol and flamed. Five wood chips (3-5 mm²) from the margins of necrotic and healthy tissue were cut from each analysed segment using a sterile scalpel and plated onto Petri dishes Malt Extract Agar (MEA) (Biokar-Diagnostics, Zac de Ther, France) supplemented with 0.5 g/L chloramphenicol.

For root isolations from each plant, root sections were cut from necrotic areas, washed under running tap water, surface-disinfested for 2 min in a 1.5% sodium hypochlorite solution, and washed twice with sterile distilled water. Ten (10) small root pieces were plated onto two Petri dishes with potato dextrose agar (PDA) (Biokar-Diagnostics, Zac de Ther, France) supplemented with 0.5 g/L of streptomycin sulphate (Reig Jofré S.A., Barcelona, Spain) (PDAS).

Plates were incubated for 15-28 d at 22 °C in the dark. During that time, they were inspected daily for the emergence of fungi. Emerging fungi were isolated in pure culture and grown on PDA prior to morphological and molecular identification.

P. chlamydospora was identified by conidiophore morphology, conidial size and shape, and its cultural characteristics on PDA and MEA. Morphological characters that were useful in distinguishing Phaeoacremonium species included conidiophore morphology, phialide type and shape, the size of hyphal warts and colony characters and pigment production on MEA, PDA and oatmeal agar (OA) (60 g oatmeal; 12.5 g agar; Difco, France) [21].

Species of the Botryosphaeriaceae, which were recognized by their fast-growing colonies with grey mycelium, were identified by colony and conidial morphology [22]. Isolates were transferred from PDA to water agar medium with two pieces of sterilized pine needles to induce sporulation and pycnidia formation. Isolates were examined weekly for formation of pycnidia and conidia. Conidial morphology (cell wall, shape, colour and presence or absence of septa) from pycnidia was recorded.

Cylindrocarpon-like anamorphs were identified by macroscopic characteristics including colony texture, brown to orange colour and the shape of the growing margin on PDA.

2.2.2 DNA Extraction, Amplification and Sequencing

Isolates obtained from young plants were analysed by molecular approaches. For that purpose, genomic DNA of the isolates was extracted from pure cultures grown on PDA for two to three weeks at 25 °C in the dark. Total DNA was extracted using the E.Z.N.A.® Plant DNA Kit (Omega Bio-tek, Doraville, GA, USA) following manufacturer’s instructions.

P. chlamydospora was identified by polymerase chain reaction (PCR) using primers Pch1-Pch2 [23], and confirmed by sequencing the ITS region of DNA using the primers ITS1F and ITS4 [24].

Phaeoacremonium spp. were identified by sequence analysis of regions of two genes, the β-tubulin gene using primer sets T1 [25] and Bt2b [26], and the 5.8S nuclear ribosomal RNA gene and the flanking internal transcribed spacers (ITS1 and ITS2), amplified using primers ITS1 and ITS4 [27]. PCR amplifications were done according to Groenewald et al. [28].

Identification of Botryosphaeriaceae species was confirmed by analysis of elongation factor 1-α gene, amplified using EF1-728F and EF1-986R primers [29].

For Cylindrocarpon-like anamorphs identification, the histone3 gene was amplified with the CYLH3F/CYLH3R primer pair [30]. Each amplification reaction consisted of 2.5 μL of 10× PCR buffer, 2.5 μL of 2.5 mM MgCl₂, 2.5 μL of dNTPs mix (0.2 mM of each dNTP), 1 μL of 0.4 mM of each primer, 0.2 μL of Taq DNA polymerase (Canvax Biotech, Córdoba, Spain) at 1 U/μL and 1 μL of fungal genomic DNA for a final volume of 25 μL. The reaction mix was denatured at 94 °C for 3 min, followed by 35 cycles of denaturing at 94 °C for 30 s,
annealing at 34 °C for 30 s and extension at 72 °C for 50 s, followed by a final extension step of 72 °C for 10 min.

Ten microlitres (10 μL) of each amplicon was analysed by electrophoresis at 130 V for 30 min in 1% agarose gels in 1× TBE buffer (40 mM Tris, 20 mM boric acid, 1 mM EDTA, pH 8.0). The gels were stained with RedSafe™ solution (Intron Biotechnology, Korea) and visualized under ultraviolet light. The PCR products were sequenced in reverse direction by the Macrogen Sequencing Service (Amsterdam, the Netherlands).

3. Results and Discussion

All plants analysed in this work showed typical internal and external decline symptoms. In cross sections characteristic necrotic lesions were observed in all, young and mature, plants analysed. The results of the isolation from the grapevine plants and fungal identification are shown in Table 1. In 96.85% of samples, at least one grapevine trunk pathogen was isolated.

For the young plant analysis, black-foot pathogens were the predominant fungal group, being isolated in 86.8% of all vineyards sampled. *P. chlamydospora*, *P. minimum* and *P. iraniamum* fungi commonly associated with Petri disease, were isolated from 39.6% of all vineyards. In addition, five species belonging to the family Botryosphaeriaceae were detected. They were isolated from 28.4% of all vineyards.

*Cylindrocarpon*-like anamorphs were the most frequent isolates from mature plants, too, being detected in 96.88% of the samples. Esca and Botryosphaeria dieback pathogens were isolated from more than 30% of the samples.

Distribution of these pathogens in the plant is shown in Fig. 1. In young plants, *Cylindrocarpon*-like anamorphs, were mainly isolated from roots (98.76%) and the graft union (60.22%). Species of the Botryosphaeriaceae family were detected mainly in the trunk (48.44%). Fungi associated with Petri disease (*P. chlamydospora* and *P. minimum*) were found in all analysed parts in greater proportions than 10%, except in roots, where the relative distribution of these fungi cannot reach the 5%. For the mature plants analysis, *Cylindrocarpon*-like anamorphs, were mainly isolated from roots (98.45%). *P. chlamydospora* was isolated from graft union, trunk and cordons in greater proportions than 40%. *P. minimum* was isolated mainly from cordons (31.03%) and *Botryosphaeria* species from the trunk (44.44%).

**Table 1** Fungal pathogens isolated from symptomatic young and mature grapevine plants.

| Fungal species                  | % detection on samples | % of positive samples |
|---------------------------------|------------------------|-----------------------|
| *Cylindrocarpon*-like anamorphs | 72.16%                 | 87.94%                |
| Young plants                    | 75.3%                  | 86.8%                 |
| Mature plants                   | 52.71%                 | 96.88%                |
| *Phaeomoniella chlamydospora*   | 10.73%                 | 42.9%                 |
| Young plants                    | 8.82%                  | 39.6%                 |
| Mature plants                   | 22.48%                 | 68.75%                |
| *Phaeosacremonium minimum*      | 7.53%                  | 18.79%                |
| Young plants                    | 7.18%                  | 16.8%                 |
| Mature plants                   | 9.69%                  | 34.36%                |
| Botryosphaeriaceae              | 8.93%                  | 29.43%                |
| Young plants                    | 8.44%                  | 28.4%                 |
| Mature plants                   | 12.02%                 | 37.3%                 |
| *Fomitiporia mediterranea*      | 0.65%                  | 0.49%                 |
| Young plants                    | 0.25%                  | 0.8%                  |
| Mature plants                   | 3.1%                   | 1.25%                 |
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Botryosphaeria dieback, which involves over 20 species of the Botryosphaeriaceae family, has been reported mainly in mature plants [31]. However, several species of Botryosphaeriaceae have also been isolated from young plants showing decline symptoms [32]. For young plants, five species of the Botryosphaeriaceae family had been detected. It is accepted that for mature plants these species are mainly spread by airborne spores and by pruning wounds, however they have been isolated in young plants during the propagation process [33]. Diplodia seriata was the prevalent species, being detected in 21.18% of all the young samples, while Neofusicoccum parvum, N. luteum, Botryosphaeria dothidea and Lasiodiplodia theobromae were detected at a lower frequency. In both analyses, these species were found mainly in the trunk. Traditionally the Botryosphaeriaceae family was associated with mature plants, however Giménez-Jaime et al. [17] showed that these species (especially D. seriata) were frequently isolated from propagation material in all steps including, grafting and callusing.

P. chlamydospora is responsible for significant economic losses for the grape industry, being regarded as one of the most important fungal pathogens associated with esca (mature plants) and Petri disease (young plants) [8, 10]. In this case, P. chlamydospora was isolated from 42.9% of all the samples, being detected in greater proportions from mature plants (68.75%). For mature plants, its distribution was similar in all the evaluated parts (excluding roots), being isolated most frequently from the cordons. However, in young samples, it was isolated mainly from the cordons. P. chlamydospora can infect plants through pruning wound and the root system [34]. This fact could explain its distribution especially in the cordons, but also in all the evaluated parts. Several species of the Phaeoacremonium genus are associated with the same diseases than P. chlamydospora, being P. minimum the most common species. In the
analysed samples, two *Phaeoacremonium* species were found, *P. minimum* and *P. iraniamum*. In young plants *P. minimum* was detected in 18.79% of the samples, being found in all analysed parts (excluding roots) with similar isolation rates. In mature plants *P. minimum* was detected in greater proportions (34.36%), being isolated mainly from the cordons. Although it has been shown that *P. minimum* can infect and colonise grapevine roots, its main infection way is through aerial dispersion [35].

*Cylindrocarpon*-like anamorphs are by far the most frequent species detected in this study, being present in 87.94% of all the samples. It is striking the high number of isolates obtained from mature plants (52.71% of the total), although these species are associated with young grapevines decline. They were mainly isolated from roots and the graft union, because of the fact that these fungi are common soil inhabitants [36]. Due to the high number of *Cylindrocarpon*-like anamorphs detected and the impossibility to analyse all of them, 32 isolates obtained from seven different vineyards, were selected and were amplified with the primers CYLH3F and CYLH3R in order to determine the identity of the most frequent species in Castilla-La Mancha region. A PCR fragment of 500 bp was obtained from each isolate. The identity of each amplicon was established by comparison with the GenBank database. As a result, 23 isolates were classified into four species of the *Dactylonectria* genus: *D. alcacerensis*, *D. macrodidyma*, *D. novozelandica* and *D. torresensis*. The remaining nine isolates were identified as *I. liriodendri*. *D. torresensis* was the most common species representing 40.6% of all of them, followed by *I. liriodendri* with 28.1%. These results are consistent with previous report [37]. Both species have been reported as causal agent of black-foot disease in Spain and in other wine producing countries such as Portugal, South Africa and Italy [6, 8, 19].

*D. novozelandica*, *D. alcacerensis* and *D. macrodidyma* presented a frequency of 15.6%, 9.4% and 6.3%, respectively. Different black-foot disease fungal species were identified, their number and percentage, and the corresponding number of positive vineyards are shown in Table 2.

These results show that in mature and young grapevines the same pathogens can be detected. This fact could suggest a common origin. Several studies have emphasized the role of the propagation material in GTDs dispersion [16, 38]. Botryosphaeriaceae species (specially *D. seriata*), *P. chlamydospora*, *P. minimum* and *Dactylonectria* and *Ilyonectria* species have been detected in nurseries [19]. This confirms that the propagation process of grapevine plants could be an important source of inoculum for Petri disease and black-foot disease pathogens [39, 40]. Moreover, the nursery soil has also been proved as an important inoculum source for black-foot pathogens and to a lesser extent for Petri disease pathogens [20].

### 4. Conclusions

This work reveals the GTDs pathogens that play a major role in the decline of young and mature grapevines in Castilla-La Mancha. In the last years, an increase in the incidence of GTDs has been reported worldwide, especially for young vines. The results obtained confirmed the high incidence of *P.*
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*chlamydospora*, *Phaeoacremonium* spp. (esca and Petri disease), *D. seriata* (Botryosphaeria dieback) and *Cylindrocarpon*-like anamorphs (black-foot disease) in vineyards from Castilla-La Mancha region. All these fungi and their associated diseases have been previously reported in other wine producing countries. Considering these data, it can be stated that *Cylindrocarpon*-like anamorphs are the most frequent GTDs pathogens in Castilla-La Mancha region. It is important to point out the high incidence of *Cylindrocarpon*-like anamorphs in mature plants.

The common origin suggested for pathogens in mature and young plants, and the lack of effective control solutions against these fungi, make it necessary to implement an integrated disease management strategy which starts in nurseries. This strategy includes cultural, physical, chemical, biological and other measures. The combination of all these measures could provide an efficient approach to reduce infections in nurseries and get better results in infected vineyards.

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