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Fungal grapevine trunk pathogens associated with Syrah decline in Spain

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Summary. Syrah decline has been increasingly seen and reported in many vineyards worldwide. In recent years, an increase in samples of *Vitis vinifera* cv. Syrah showing general decline has also been noted in Spain. Sixty-two samples of Syrah grafted grapevines with such symptoms were collected from grapevine nurseries and young vineyards between 2007 and 2009 and subjected to fungal isolation. Species were identified with morphological and molecular methods. Species recovered included *Phaeoacremonium,* Botryosphaeriaceae and *Cylindrocarpon*, as well as *Pa. chlamydospora* and *Cadophora luteo-olivacea*. The study demonstrates that fungal pathogens should be considered potential factors associated with Syrah decline.

Key words: Botryosphaeriaceae, *Cadophora luteo-olivacea*, *Cylindrocarpon* spp., *Phaeoacremonium* spp., *Phaeomoniella chlamydospora*.

Introduction

Since the early 1990s, Syrah vine decline symptoms have been increasingly observed and reported in many vineyards worldwide (Rieger, 2008). Various names have been associated with these decline problems that vary in their symptoms and location. The condition is known as Syrah decline in France (Renault-Spilmont and Bourisquot, 2002; Renault-Spilmont *et al*., 2003), and also in California's North Coast and Sierra Foothills, where similar symptoms have been found (Battany *et al*., 2004). It has been called Syrah disorder in the middle part of California's coastline (Battany *et al*., 2004). South Africa uses both Shiraz (syn. Syrah) disease and Shiraz decline (Carstens, 1999; Spreeth, 2005), and there is an Australian Shiraz disease in Australia (Habili and Randles, 2004).

In France, Syrah decline is characterized by swelling and cracking of the graft union followed by early leaf reddening, resulting in young vine death after a four to ten year period (Battany *et al*., 2004). Symptoms rarely develop within the first five or six years of planting, although vines as young as three years old have been affected (Renault-Spilmont *et al*., 2003). Leaves on affected vines may turn light-green or yellow in the spring prior to reddening later in the season. Fruit fails to become fully ripened, typified by poor color and sugar development. When examined after bark removal, affected graft unions exhibit deep grooves in the wood (Stamp, 2004).

Symptoms in young grapevines growing in California's mid coast area included mild to severe vertical cracking of the trunk, the interveinal areas of the leaves turning burgundy, leaf-margin reddening and necrosis, scorching, swelling at the graft union, and failure of the fruit to ripen fully (Battany *et al*., 2004). Although the symptoms of af-

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fected vines in France and California are similar, several authors have tried to distinguish between them. Stamp (2004) reported that affected Syrah vines in California develop red leaves, but that unlike in France, veins may also remain green, and graft unions may or may not become cracked and swollen. Walker (2005) observed that Californian vines seemed to decline faster than vines in France. Recently, Rieger (2008) reported that Californian vines could also have cracks on the arms and cordons, but those symptoms were not seen in France.

In South Africa, Shiraz decline shows symptoms similar to those in other countries; however, it is only found in Shiraz clone 99, imported from France in 1982, and clone 99B, cultured in South Africa from Shiraz 99 in 1986 (Spreeth, 2005). Young vines affected with Shiraz disease in South Africa and Australia develop symptoms near the end of the growing season with droopy canes that never mature fully, cross-sections showing weak wood and excessive phloem development, stunted and zigzag growth of canes and leaves with leafroll-like symptoms (Carstens, 1999; Habili, 2006).

To date, studies have failed to show any useful correlation between Syrah decline or disorder and soil type, clone, viral pathogens or the environment. After surveys carried out in California, Battany *et al*. (2004) reported that symptoms in problem vineyards could be attributed to a number of specific factors, including frost damage of young vines, water stress, and poor planting and training techniques. Studies on Syrah decline in France indicated that Syrah may have more problems in establishing a good graft union than other varieties (Renault-Spilmont and Boursiquot, 2002). Renault-Spilmont *et al*. (2007) excluded phytopathogenic bacteria and phytoplasma infection as a possible cause for Syrah decline in French vineyards. Several virus variants have been associated with Shiraz disease in Australia and South Africa. *Grapevine virus A* (GVA) was detected in both South African and Australian Shiraz-affected vines (Symons and Habili, 2000; Goszczynski and Jooste, 2003; Habili and Randles, 2004). *Apple stem pitting virus* (ASPV) was also found in clone 99B in South Africa (Goszczynski, 2007). Although research found that a number of viruses were associated with Syrah vines exhibiting Syrah decline symptoms, it was not clear whether those viruses were the direct cause of the disease. It is possible that unknown or undetectable viruses, or new strains, may exist that contribute to Syrah decline (Rieger, 2008).

Battany *et al*. (2004) reported that root stress symptoms, such as those caused by Armillaria root rot, could be attributed to Syrah decline in problem vineyards. Other fungi, including *Phomopsis*, *Verticillium* and *Alternaria,* and those involved with esca and *Eutypa,* were found associated with the graft unions of symptomatic plants; however these fungi also occurred in control plants, so that it was not possible to correlate them specifically with Syrah decline (Renault-Spilmont *et al*., 2003, 2007).

Over the last decade, the incidence of fungal grapevine trunk pathogens has increased significantly around the world, particularly in grapevine nurseries and young vineyards. Petri disease pathogens (*Phaeomoniella chlamydospora* and numerous species of the genus *Phaeoacremonium*), black-foot disease pathogens (*Cylindrocarpon* spp.) and species of Botryosphaeriaceae have been associated with grapevine decline (Mugnai *et al.*, 1999; Van Niekerk *et al*., 2004; Halleen *et al.*, 2006; Mostert *et al.*, 2006).

In recent years, an increase in the number of samples of *Vitis vinifera* var. Syrah showing symptoms of general decline has also been noted in Spain. There is no information about what grapevine trunk pathogens are associated with Syrah decline. The aim of this work was to determine the occurrence of fungal trunk pathogens in vines affected with Syrah decline.

Materials and methods

Sampling and isolation of fungi

Sixty-two samples of Syrah grafted grapevines obtained from grapevine nurseries and young vineyards (<7 years) in Spain between 2007 and 2009 were subjected to fungal isolation. Symptoms included poor early growth and reduced vigour. At least 5–7 plants were analyzed per sample.

Rootstocks, graft unions and scions were examined. Segments were cut from affected areas, washed under running tap water, surface-disinfected for 1 min in a 1.5% sodium hypochlorite solution, and washed twice with sterile distilled water. Small pieces of discolored or decayed tissues were plated on malt extract agar (MEA) (Oxoid Ltd., Basingstoke, England) supplemented with 0.5 g L^{-1} of streptomycin sulphate (MEAS) (Sigma-Aldrich, St. Louis, MO, USA). Plates were incubated at 25ºC in the dark for 14 to 21 days, and all colonies were transferred to 2% potato dextrose agar (PDA; Biokar-Diagnostics, Zac de Ther, France). They were single-spored prior to morphological and molecular identification with the serial dilution method (Dhingra and Sinclair, 1995).

Fungal identification

Morphological identification

Morphological characters to distinguish species of *Phaeoacremonium* included conidiophore morphology,

phialide type and shape, 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) (Mostert *et al.*, 2006). *Pa. chlamydospora* was identified by conidiophore morphology, conidial size and shape, and its cultural characteristics on PDA and MEA (Crous and Gams, 2000).

Species of Botryosphaeriaceae were identified by colony and conidial morphology (Phillips, 2006). In order to enhance sporulation, cultures were placed with sterilized pine needles on 2% water agar (WA; Biokar-Diagnostics) at 25°C with a 12-h day (Philips TDL18W/33) (Slippers *et al*., 2004). Isolates were examined weekly for formation of pycnidia and conidia. Conidial morphology (cell wall, shape, color, and presence or absence of septa) from pycnidia was recorded.

Species of *Cylindrocarpon* were identified by macroscopic characters such as colony texture, color, and the type of the growing margin on PDA. Colonies grown on PDA were incubated for a further 20 days to determine the presence/absence of chlamydospores. Conidia size was also measured on Spezieller Nährstoffarmer Agar (SNA) with the addition of a 1×1 cm piece of filter paper to the colony surface (Alaniz *et al*., 2007).

Cadophora luteo-olivacea was identified by conidiophore morphology, size of phialides and conidia, and colony characters and pigment production on MEA, PDA and OA (Gams, 2000; Harrington and McNew, 2003).

DNA isolation and sequencing

Fungal mycelium and conidia from pure cultures grown on PDA for 2 to 3 weeks at 25ºC in the dark were scraped and mechanically disrupted by grinding to a fine powder under liquid nitrogen using a mortar and pestle. Total DNA was extracted using the E.Z.N.A. Plant Miniprep Kit (Omega Bio-tek, Doraville, GA, USA) following manufacturer's instructions. DNA was visualized on 0.7% agarose gels stained with ethidium bromide and was stored at -20ºC.

Phaeoacremonium species were identified with Restriction Fragment Length Polymorphism-Polymerase Chain Reaction (RFLP-PCR) (Aroca and Raposo, 2007) and confirmed by sequence analysis of the β-tubulin gene using primer sets T1 (O'Donnell and Cigelnik, 1997) and Bt2b (Glass and Donaldson, 1995), and by comparison with the polyphasic, online identification system for *Phaeoacremonium* species recognition (http://www.cbs.knaw.nl/phaeoacremonium/biolomics.aspx) developed by Mostert *et al*. (2006). *Pa. chlamydospora* was detected by PCR using primers Pch1-Pch2 (Tegli *et al*., 2000). Identification of Botryosphaeriaceae species was confirmed by analysis of elongation factor 1-α gen amplified using EF1-728F and EF1-986R primers (Carbone and Kohn, 1999). Identification of *Cylindrocarpon* species was confirmed by a multiplex PCR system using a set of three pairs of specific primers (Alaniz *et al*., 2009). Identification of *Ca. luteo-olivacea* isolates was confirmed by analysis of the ITS region of DNA amplified using the fungal universal primers ITS1F and ITS4 (Gardes and Bruns, 1993). PCR products were purified with the High Pure PCR Product Purification Kit (Roche Diagnostics, Mannheim, Germany) and sequenced in both directions by the DNA Sequencing Service of the Universidad Politécnica de Valencia-CSIC.

Results

Sixty-two samples collected from 10 provinces in Spain were studied (Table 1). Fungal trunk pathogens were isolated from 47 samples (75.8%). Several Petri disease pathogens (*Phaeoacremonium aleophilum*, *Pm. cinereum*, *Pm. inflatipes*, *Pm. iranianum*, *Pm. parasiticum* and *Pa. chlamydospora*), Botryosphaeriaceae species (*Botryosphaeria dothidea*, *Diplodia mutila*, *D. seriata*, *Dothiorella sarmentorum* and *Neofusicoccum parvum*), black foot disease pathogens (*Cylindrocarpon macrodidymum* and *Cy. liriodendri*) and *Ca. luteo-olivacea* were isolated (Table 1).

As regards the vine portions from which the pathogens were isolated, species of *Phaeoacremonium* were isolated from the rootstocks (all rootstock samples contained *Phaeoacremonium* spp.) and from the graft unions (33.3%). *Pa. chlamydospora* was isolated from all the rootstock samples, from the graft unions (33.3%) and from the scion cultivars (33.3%). Species of Botryosphaeriaceae were isolated from the rootstocks (23.1%), the graft unions (61.5%) and the scion cultivars (61.5%). Species of *Cylindrocarpon* were isolated only from the rootstocks, but from all of them. *Ca. luteo-olivacea* was isolated from the rootstocks (100%) and the graft unions (66.6%) (Table 1).

The proportion of fungal trunk pathogens isolated, as a percentage of the positive samples obtained, is shown in Figure 1. *Pa. chlamydospora* was the most frequently isolated species (36.2% of positive samples). Species of *Phaeoacremonium* were isolated in 36.1% of positive samples (21.3% *Pm. aleophilum*, 6.4% *Pm. iranianum*, 4.2% *Pm. parasiticum*, 2.1% *Pm. cinereum* and 2.1% *Pm. inflatipes*). Species of Botryosphaeriaceae were isolated in 31.9% of positive samples (12.8% *D. seriata*, 12.8% *N. parvum*, 2.1% *B. dothidea*, 2.1% *D. mutila* and 2.1% *Do. sarmentorum*). Species of *Cylindrocarpon* were isolated in 19.1% of positive samples (14.9% *Cy. macrodidymum* and 4.2% *Cy. liriodendri*). *Ca. luteo olivacea* was isolated in 14.9% of positive samples.

Table 1. Geographical origin, number of samples analyzed, fungal trunk pathogens isolated and plant portion from which pathogens were recovered in Syrah grafted grapevines collected between 2007 and 2009.

Province	Number of samples		Fungal trunk pathogens isolated[b]	Samples[c] No.	Isolation area[d]		
	Total	Positive[a]			Rootstock	Graft union	Scion
Albacete	5	4	*Botryosphaeria dothidea*	1	-	+	-
			Diplodia seriata	2	-	+	+
			Neofusicoccum parvum	1	-	+	+
Almería	1	1	*Phaeomoniella chlamydospora*	1	+	-	-
Badajoz	8	8	*Cadophora luteo-olivacea*	3	+	+	-
			Cylindrocarpon macrodidymum	1	+	-	-
			D. mutila	1	+	-	-
			D. seriata	1	+	-	-
			Phaeoacremonium aleophilum	1	+	-	-
			Pm. cinereum	1	+	-	-
			Pm. inflatipes	1	+	-	-
			Pm. iranianum	1	+	-	-
			Pm. parasiticum	2	+	+	-
Ciudad Real	11	6	*Cy. macrodidymum*	1	+	-	-
			Cy. liriodendri	1	+	-	-
			N. parvum	1	-	+	+
			Pm. aleophilum	1	+	-	-
			Pa. chlamydospora	5	+	+	-
Cuenca	10	7	*Cy. liriodendri*	1	+	-	-
			Cy. macrodidymum	2	+	-	-
			D. seriata	1	-	-	+
			Pm. aleophilum	3	+	-	-
			Pa. chlamydospora	2	+	-	+
Huesca	1	1	*N. parvum*	1	-	-	+
Islas Baleares	3	3	*D. seriata*	1	+	-	+
			Pa. chlamydospora	3	+	-	+
Toledo	16	11	*D. seriata*	1	-	+	-
			Dothiorella sarmentorum	1	-	+	-
			N. parvum	2	-	+	+
			Pm. aleophilum	3	+	+	-
			Pm. iranianum	1	+	+	-
			Pa. chlamydospora	4	+	-	-
Valencia	3	3	*Ca. luteo-olivacea*	3	+	+	-
			Cy. macrodidymum	2	+	-	-
			Pm. aleophilum	1	+	+	-
Zaragoza	4	3	*Ca. luteo-olivacea*	1	+	-	-
			Cy. macrodidymum	1	+	-	-
			N. parvum	1	-	+	+
			Pm. aleophilum	1	+	-	-
			Pm. iranianum	1	+	-	-
			Pa. chlamydospora	2	+	+	-
Total	62	47					

[a] Samples from which fungal trunk pathogens were isolated.
[b] Fungal trunk pathogens were identified by means of morphological and molecular methods.
[c] Samples from which each pathogen was detected.
[d] +/-; presence/absence of each pathogen in the different isolation areas.

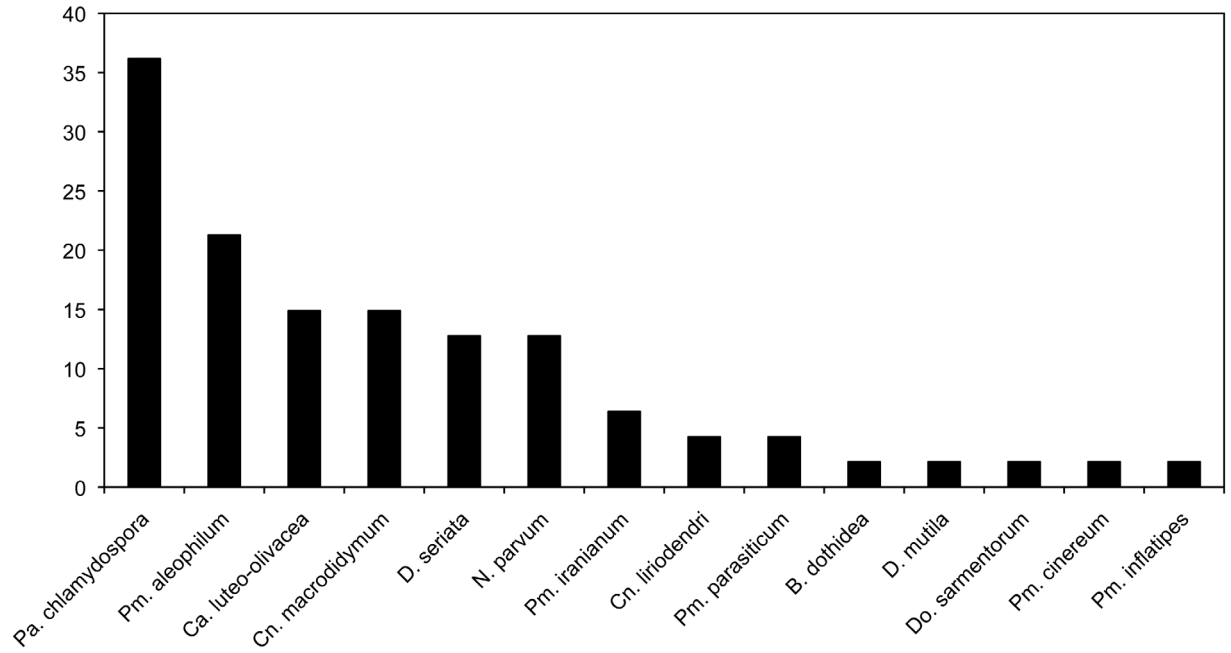

Figure 1. Isolation percentages of fungal trunk pathogens on the positive samples.

Discussion

The study shows the high incidence and diversity of fungal trunk pathogens found in Syrah grafted grapevines in Spain. The fungi most frequently isolated from symptomatic plants were *Pa. chlamydospora* and *Pm. aleophilum*, which are also the most commonly isolated species from young vines showing a general decline (Mugnai *et al*., 1999). Numerous other species of the genus *Phaeoacremonium* have also been associated with grapevine declines in grape-growing regions throughout the world, although their importance is thought to be minor (Mostert *et al*., 2006; Essakhi *et al*., 2008; Gramaje *et al*., 2009). *Pa. chlamydospora* and *Phaeoacremonium* spp. were isolated mainly from grapevine rootstocks, and, in a few cases, from graft unions or scion cultivars. Several authors have reported that these pathogens are more frequent in grapevine rootstocks than in scion cultivars (Aroca *et al*., 2006); however, pathogenicity studies have found that both *Pa. chlamydospora* and *Pm. aleophilum* readily infected pruning wounds on *Vitis vinifera* cultivars following inoculation with conidia (Larignon and Dubos, 2000; Eskalen *et al*., 2007).

The most frequently isolated Botryosphaeriaceae spp. were *D. seriata* and *N. parvum*. These species, together with *B. dothidea*, are considered the most common species associated with the grapevine decline syndrome in Spain (Armengol *et al*., 2001; Aroca *et al*., 2006). Species of Botryosphaeriaceae were isolated mainly from graft unions and scion cultivars. In Portugal, *B. dothidea, D. seriata* and *N. parvum* were also isolated from failed graft unions of young plants (Phillips, 2002). This researcher reported that these early infections could remain latent until the young grapevines were exposed to stress or conditions favoring disease development, which might then lead to the decline and sometimes death of the young grapevines. More recently, a phenotypic and molecular characterisation of isolates from grapevine propagating materials, and from young and mature vine plants with decline symptoms, black streaks, necroses or esca-like symptoms allowed the identification of six Botryosphaeriaceae species: *B. dothidea*, *D. seriata, D. mutila, Dothiorella viticola, N. luteum* and *N. parvum* (Rego *et al.,* 2009).

Species of Botryosphaeriaceae have traditionally been considered wound pathogens (Van Niekerk *et al*., 2006); however, Slippers and Wingfield (2007) reported that these fungi also directly infected plants without wounds, penetrating through the lenticels, stomata and other natural openings.

Species of *Cylindrocarpon* were also commonly isolated in this study, *Cy. macrodidymum* being the most frequent. This is consistent with Alaniz *et al*. (2007),

who found that this species was also the most common *Cylindrocarpon* species associated with black foot disease in Spain. As regards the vine portion from which the pathogen was recovered, both *Cy. liriodendri* and *Cy. macrodidymum* isolates were only collected from the rootstocks. Species of *Cylindrocarpon* are common soil inhabitants, which produce black discoloration and necrosis of the wood tissues, developing from the base of the rootstocks (Halleen *et al.*, 2006).

The vascular pathogen *Ca. luteo-olivacea* was common in Syrah-affected vines. Recently, *Ca. luteo-olivacea* has been identified in grapevines showing decline symptoms in California, South Africa and New Zealand (Rooney-Latham, 2005; Halleen *et al*., 2007; Manning and Mundy, 2009). Halleen *et al*. (2007) confirmed that artificially inoculated *Ca. luteo-olivacea* colonized, survived and caused lesions in grapevine pruning wounds and trunks and was therefore a potential pathogen.

Diverse fungal trunk pathogens were isolated from symptomatic Syrah vines in this study. Several researchers have suggested that fungi involved in wood diseases play only a secondary role in increasing or quickening the decline of already weakened plants, and in this way inducing necrosis leading to plant death (Stamp, 2004; Renault-Spilmont *et al*., 2007). It has also been hypothesized that an unknown factor, possibly pathological (viral, bacterial or fungal), or environmental, or genetic (related to incompatibility), causes abnormal cambial activity in healthy vines thereby causing fissures and lesions in graft union tissues (Stamp, 2004). Subsequently, a second (unknown) factor or factors, possibly nutritional imbalance, water stress, overcropping or fungal pathogen pressure, induce further stress, leading to the development of red foliage and the decline and death of the vine over a relatively short period (Renault-Spilmont and Boursiquot, 2002; Battany *et al*., 2004).

Syrah decline appears to be very complex and probably involves numerous factors. This study demonstrated that fungal pathogens should be considered a potential factor in Syrah decline. However, further research is needed to better understand the role of trunk disease pathogens in this syndrome.

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