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Evaluation of the grapevine nursery propagation process as a source of *Phaeoacremonium* spp. and *Phaeomoniella chlamydospora* and occurrence of trunk disease pathogens in rootstock mother vines in Spain

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Abstract Five commercial nurseries were sampled in 2007 to evaluate the grapevine nursery propagation process as a source of Petri disease pathogens (*Phaeoacremonium* spp. and *Phaeomoniella chlamydospora*). Samples were taken at four stages of the propagation process: pre-grafting hydration tanks, scissors used for cutting buds, grafting machines and peat used to promote root development. All samples were analysed using two different techniques: nested PCR using specific primers for *Phaeoacremonium* spp. (Pm1/Pm2) and *Pa. chlamydospora* (Pch1/Pch2); and fungal isolation by culturing on semi-selective medium. Either *Phaeoacremonium* spp. or *Pa. chlamydospora* were detected at any of these stages, and more importantly they were viable since they were detected by isolating on culturing medium. Additionally, the importance of grapevine rootstock mother fields as sources of inoculum in the nurseries was studied. Fourteen grapevine rootstock mother fields were surveyed in 2006 and 2007 for the occurrence of fungal trunk pathogens. A total of 16.4% and 30% of the plants sampled in 2006 and 2007, respectively were infected. Petri disease pathogens (*Pa. chlamydospora*, *Phaeoacremonium aleophilum*, *Pm. parasiticum*) and several Botryosphaeriaceae species (*Neofusicoccum parvum*, *Botryosphaeria dothidea*, *Lasiodiplodia theobromae*, *N. australe*, *N. mediterraneum* and *N. vitifusiforme*) and *Phomopsis viticola* were isolated. This is the first time *N. mediterraneum* has been isolated from grapevines and the first report of *N. australe*, *N. mediterraneum* and *N. vitifusiforme* in Spain. This work shows that grapevine rootstock mother plants and the propagation process of grapevine plants should be considered as important sources of inoculum for fungal trunk pathogens, and especially of Petri disease pathogens.

Keywords Botryosphaeriaceae · Petri disease · *Phomopsis* spp. · *Vitis vinifera*

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Introduction

Worldwide, a drastic increase in grapevine mortality has been noticed in new plantings over the last few years. Surveys of diseased young grapevine plants suggest that grapevine propagating material used for new grafted plants is mainly infected with fungi involved in Petri disease (caused by *Phaeomoniella chlamydospora* and several *Phaeoacremonium* species) and also by other fungal trunk pathogens (Ridgway et al. 2002; Halleen et al. 2003; Fourie and Halleen 2004a; Aroca et al. 2006; Giménez-Jaime et al. 2006; Retief et al. 2006; Whiteman et al. 2007; Zanzotto et al. 2007). Moreover, it has been suggested that infection of new plants may also occur in the grafting process in nurseries (Mugnai et al. 1999; Halleen et al. 2003). Surveys performed in French, Italian, New Zealand and South African grapevine nurseries showed that *Pa. chlamydospora* DNA was detectable in water from pre-storage rehydration tanks, pre-grafting hydration tanks, washings from grafting tools, callusing media or vineyard soils (Whiteman et al. 2004, 2007; Damm and Fourie 2005; Retief et al. 2006; Pollastro et al. 2009; Vigues et al. 2008).

Several surveys of young vineyards have been carried out during recent years in Spain and the results reported typical trunk disease pathogens occurring all over the main growing areas (Armengol et al. 2001; Aroca et al. 2006; Giménez-Jaime et al. 2006). Fungal species such as *Pa. chlamydospora*, *Pm. aleophilum* and *Pm. parasiticum* (causal agents of Petri disease), *Cylindrocarpon macrodidymum* and *C. liriodendri* (causal agents of black foot disease) (Alaniz et al. 2007), and several species of Botryosphaeriaceae (*Diplodia seriata*, *Neofusicocum parvum* and *Botryosphaeria dothidea*) were isolated from plants used in new plantings. These results suggest that new plants were infected during the propagation process in nurseries and that even the planting material used in the propagation process might be infected with these pathogens (Aroca et al. 2006).

The grafting process in Spanish nurseries starts in December when grapevine rootstock mother plants and scion cuttings are harvested. These cuttings are stored at 5–6°C and high relative humidity (RH) (>90%); in February and March, cuttings are soaked in hydration tanks for four days. An average tank has a capacity of 50,000 l and accommodates 30,000 cuttings. After cuttings are taken out of the tank, the tank is cleaned before a new batch is introduced. Buds of hydrated canes are manually removed with scissors, and rootstock and scion cuttings are mechanically-grafted with an omega-cut grafting machine. The graft union is sealed by dipping in a melted fungicide-impregnated wax formulation. Grafted plants are placed upright in plastic boxes with a 10 cm-peat bed and stored at 26–27°C and 80% RH for 20 days to promote callus formation and root development. Following successful callusing, graftlings are transplanted in nursery fields for one growing season to allow growth of roots and buds from the cultivar. After this, plants are uprooted and stored at 5–6°C until distribution to farmers.

Surveys of the nursery propagation process as a source of Petri disease pathogens have been focused mainly on the detection of *Pa. chlamydospora* (Ridgway et al. 2002; Retief et al. 2006; Whiteman et al. 2004, 2007; Pollastro et al. 2009; Edwards et al. 2007), probably because there are available well-validated species-specific primers (Pch1 and Pch2) to detect this pathogen in soil, water and the plant (Tegli et al. 2000). Conversely, the detection of *Phaeoacremonium* species is not easy. Taxonomy of this genus has been repeatedly reviewed, with new species described in recent years. Several species of *Phaeoacremonium* have been associated with Petri disease of grapevine, although their pathogenicity has not been demonstrated for all of them (Aroca et al. 2009). Recently, Aroca and Raposo (2007) designed a pair of primers (Pm1 and Pm2) to detect any of the 13 *Phaeoacremonium* species described prior to 2007. They also developed a Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) method to identify any of those species, based on the band pattern obtained by digestion with restriction enzymes. Application of this method on DNA from different grapevine samples allowed the occurrence of some *Phaeoacremonium* species, not previously reported in Spain, to be detected (Gramaje et al. 2007; Aroca et al. 2008a; Gramaje et al. 2008). It also showed the existence of new species that did not match any existing banding pattern, giving rise to the description of new species (Gramaje et al. 2009a) and demonstrating that these primers are valid even for those *Phaeoacremonium* species described later than the design of the primers. Primers are located in the region ITS1/5.8S/ITS2 of

rDNA (Aroca and Raposo 2007), which is highly conserved for the *Phaeoacremonium* species.

In addition, results of surveys of *Pa. chlamydospora* in the grapevine nurseries are based on positive detection using PCR (Ridgway et al. 2002; Retief et al. 2006; Whiteman et al. 2004) rather than using the traditional method of culturing in a growth medium. Growth of Petri disease pathogens in an enriched medium is very slow, and their ability to compete with other fungi and bacteria arising from plant material is limited, characteristics that make the traditional method less efficient for detecting these pathogens (Aroca and Raposo 2009). However, a positive result by PCR does not necessarily imply that propagules are viable for infection. A semi-selective medium for *Pa. chlamydospora* has been described (Tello et al. 2009), but growth is still very slow.

The objective of this work was to evaluate the nursery grapevine propagation process as a potential infection source for *Phaeoacremonium* spp. and *Pa. chlamydospora* in Spain. Detection was performed by nested PCR and by culturing in a semi-selective culture medium. A survey of grapevine rootstock mother fields was also carried out to discover the importance of this plant material as potential inoculum of fungal trunk pathogens in nurseries.

Materials and methods

Sampling of grapevine nurseries

Five commercial nurseries located in Comunidad Valenciana region (central-eastern Spain) were surveyed in 2007. Samples from nurseries were taken arbitrarily at four stages of the propagation process: pre-grafting hydration tanks, washings from scissors used for cutting buds, washings from omega-cut grafting machines and peat from the callusing stage. Samples of tank water (approximately 100 ml each) were taken from the middle of the tank stirring slightly. Sampling was done in each hydration period when cuttings had been immersed for two to three days. A sample of the tap water used to fill the tanks was also collected. Scissors and omega-cut grafting machines were washed with a sterile solution of 0.2% Tween-20 at the end of one working day, and 30 to 60 ml of every washing was recovered in a sterile tube. Samples of about 500 g of peat were taken after the callusing stage for a batch of finished cuttings. One sample of peat was also taken before use. For each peat sample, a subsample of 50 g was mixed with 150 ml of a sterile solution of 1% Tween-20 and stirred for 1 h at 200 rpm; it was then filtered through a cheesecloth filter and 100 ml collected in sterile tubes. The total number of samples taken in the five nurseries was 30 from hydration tanks, 28 from scissors, 13 from omega-cut grafting machines and 18 from peat after the callusing process.

All samples were divided in two aliquots and each one was analysed for the presence of *Phaeoacremonium* spp. and *Pa. chlamydospora* using a different technique, (a) nested PCR using specific primers for *Phaeoacremonium* spp. and *Pa. chlamydospora*; and (b) fungal isolation by culturing on semi-selective medium.

Detection of *Phaeomoniella chlamydospora* and *Phaeoacremonium* spp. by nested PCR

Samples were centrifuged for 10 min at 2790 rfc (5000 rpm); supernatant was discarded and the resulting liquid sample pellets were analysed as follows. DNA was extracted with the commercial kit 'DNeasy Plant mini kit' (Qiagen, Hilden, Germany). Extracted DNA was kept at −20°C until its use in PCR amplifications. *Phaeomoniella chlamydospora* and *Phaeoacremonium* spp. were detected by PCR using primers Pch1-Pch2 (Tegli et al. 2000) and primers Pm1-Pm2 (Aroca and Raposo 2007), respectively. A nested PCR was performed to achieve more sensitivity detecting these pathogens; a first PCR was done using the universal primers ITS1-ITS4 (Gardes and Bruns 1993); it was performed in a volume of 25 μl containing 2 μl of 10X buffer, 0.2 μM of each primer, 2 mM $MgCl_2$, 0.2 mM dNTPs, 0.75 U of *Taq* polymerase, 2.5 μl of BSA and 1 μl of DNA as template (approximately 10 ng of DNA). Conditions consisted of a first denaturation phase at 94°C for 2.5 min, 35 cycles at 94°C for 15 s, 53°C for 30 s, and 72°C for 90 s, and a final extension at 72°C for 7 min. PCR product was diluted 1:200 and then, 1 μl was used as DNA template for the secondary PCR using primer pair Pm1-Pm2 for *Phaeoacremonium* spp. or Pch1-Pch2 for *Pa. chlamydospora*. Concentration of reagents in a final volume of 25 μl was: 2.5 μl of 10X buffer (Biotools, Madrid, Spain), 0.5 μM of each primer (Sigma-Aldrich, Haverhill, Suffolk, UK), 4 mM $MgCl_2$, 0.8 mM dNTPs and

1.25 U *Taq* polymerase (Biotools, Madrid, Spain). For *Phaeoacremonium* spp. optimal amplification, it was necessary to add 1.5 µl of DMSO (Dimethyl sulfoxide, Amresco, Solon, Ohio, USA). Thermal conditions were 5 min at 94°C followed by 30 cycles of 30 s at 94°C, 30 s at 57°C, 50 s at 72°C, with a final extension at 72°C for 7 min; for *Pa. chlamydospora*, 5 min at 94°C, followed by 25 cycles of 20 s at 94°C, 30 s at 64°C, 40 s at 72°C, with a final extension at 72°C for 7 min. All PCR reactions were carried out in a Perkin-Elmer 9700 Thermal Cycler (Applied Biosystems, Foster City, CA, USA). Amplified fragments were visualised under UV light after electrophoresis on 1.5% agarose gels stained with ethidium bromide and run in 1X TBE buffer (Tris-Boric-EDTA). A positive control (*Pa. chlamydospora* and *Phaeoacremonium* DNA) and a negative control (no DNA) were included in each test. Detection sensitivity of *Phaeoacremonium* by nested PCR was 0.1 pg (Aroca et al. 2009), and that of *Pa. chlamydospora* was similarly 0.1 pg (unpublished results).

Identification of fungal pathogens in semi-selective culture medium

Ten ml of raw samples were forcibly passed through 5- and 0.45-µm pore size syringe filters (Sartorius Stedium Biotech; Goettingen, Germany) attached in tandem to sterile 20-ml syringes (BD Biosciences; Madrid, Spain) as described by Eskalen and Gubler (2001). The 5-µm filters trapped debris and large fungal spores. The 0.45-µm filters trapped smaller spores, including those of *Pa. chlamydospora* and *Phaeoacremonium* spp. The filters were rinsed with 4 ml of sterile water to backwash any trapped spores. The solutions were collected in microfuge tubes and 1 ml aliquots were then spread onto a semi-selective culture medium adapted from Tello et al. (2009) for isolating *Pa. chlamydospora* and *Phaeoacremonium* spp. (2% malt extract agar (MEA) amended with streptomycin sulphate: 1.5 g l^{-1}, folpet: 0.02 g l^{-1}, and ampicillin: 0.015 g l^{-1}). Plates were air-dried in a laminar flow hood with lids partially lifted for 30 min. Plates then were sealed with parafilm and incubated at 25°C in darkness for one month. Petri dishes were observed daily for mycelial growth and re-isolation was done to prevent overgrowth by fast-growing saprophytes.

Fungal colonies were identified by morphological characters such as fruiting structures and conidia (Crous and Gams 2000; Niekerk et al. 2004; Slippers et al. 2004; Mostert et al. 2006). Identification of *Phaeoacremonium* spp. was done by PCR-RFLP (Aroca and Raposo 2007) and confirmed by the sequence analysis of the β-tubulin gene using primer sets T1 (O'Donnell and Cigelnik 1997) and Bt2b (Glass and Donaldson 1995) and comparison to the polyphasic, online identification system for *Phaeoacremonium* spp. recognition (http://www.cbs.knaw.nl/phaeoacremonium/biolomics.aspx) developed by Mostert et al. (2006).

Sampling of mother grapevine plants and identification of pathogens

Seven grapevine rootstock mother fields located in Comunidad Valenciana region (central-eastern Spain) were surveyed in June 2006 (at shoot development). Samples from twenty plants were collected in each field. In each plant, one 15 cm-long fragment was cut from the basal part of two different shoots (two fragments per plant). Plants were labelled to be re-sampled in November 2006 (after leaf fall). A second sampling was performed in June and November 2007 in another seven mother grapevine fields. Fields sampled in 2006 were of 110 Richter rootstock and only one of them was of 140 Ruggeri. In 2007, five fields were of 110 R rootstock, one field was of Selection Oppenheim 4 and one of 1103 Paulsen. Mother vine age varied from seven to 20 years. All plants were growing prostrate on the ground.

Bark of each rootstock mother-vine fragment was removed with a sterile scalpel and three thin cross sections (1–2 mm thick) were cut from both ends, the basal and apical end. These disks were immersed in 70% ethanol for 1 min and air-dried under sterile conditions. Disks were plated on 2% MEA (Conda laboratories, Torrejón de Ardoz, Madrid, Spain) amended with streptomycin sulphate (MEAS) (100 mg l^{-1}, Sigma, Steinheim, Germany) (3 disks per plate, 6 disks per fragment). All plates were incubated at 25°C in darkness, and observed daily for mycelial growth. Isolates were transferred to potato dextrose agar (PDA) (Conda laboratories, Torrejón de Ardoz, Madrid, Spain) and incubated at 25°C under black light with 16/8 h light/dark photoperiod. Fungi were morphologically identified after microscopic examination of fruiting structures and conidia (Pearson and Goheen 1994; Crous and Gams 2000; Niekerk et

al. 2004; Slippers et al. 2004; Mostert et al. 2006). Identification of some *Phomopsis* and Botryosphaeriaceae species was confirmed by the analysis of the ITS region of rDNA (ITS1/5.8S/ITS2), β-tubulin gen or elongation factor 1-α, amplified using respectively, fungal universal primers ITS1F and ITS4 (Gardes and Bruns 1993), Bt2a and Bt2b (Glass and Donaldson 1995) and EF1-728F and EF1-986R primers (Carbone and Kohn 1999). Identification of *Phaeoacremonium* spp. was performed as previously described for cultures obtained from grapevine nursery samples.

Results

Detection of Petri disease pathogens during the nursery propagation process

Petri disease pathogens were detected in different samples taken during the propagation process. *Phaeomoniella chlamydospora* and some species of *Phaeoacremonium* were detected either in hydration tanks, scissors, grafting machines, or in peat used for root development (Table 1). However, in spite of sample aliquots analysed by both methods, species detected differed. *Phaeoacremonium* spp. were detected by nested PCR in two samples from hydration tanks, in one sample from omega-cut grafting machines, and in six peat samples taken after the callusing process (Table 1). In contrast, *Pm. aleophilum* and *Pm. parasiticum* were detected in two samples from scissors by the method of culturing on semi-selective medium. Identification of the species of *Phaeoacremonium* detected by the PCR method was not achieved. *Phaeomoniella chlamydospora* was detected by nested PCR in one sample from grafting tools and also in one peat sample taken after the callusing process when incubated on a semi-selective medium. None of these pathogens were detected in tap water and peat taken before plant processing by both methods.

Isolation and identification of pathogens in rootstock mother-vines

A total of 16.4% out of 140 plants sampled in 2006 were infected with some fungal trunk disease pathogens, while in 2007 these pathogens were isolated from 30% of the plants (out of 140 sampled plants). There were six plants infected in the first survey done in June 2006 (4.3%), and 18 plants infected in November (13.6%) (Table 2). In June 2006, *Pm. aleophilum*, Botryosphaeriaceae isolates and *Diaporthe helianthi* were respectively isolated from 2.1%, 1.4% and 0.7% of the plants. In November 2006, Botryosphaeriaceae isolates, *Pa. chlamydospora* and *Pm. parasiticum* were isolated from 10.7%, 2.9% and 0.7% of the plants respectively. One of these plants was simultaneously infected by *Pa. chlamydospora* and *Pm. parasiticum*. *Phaeoacremonium aleophilum* was not isolated from

Table 1 Detection of species of *Phaeoacremonium* and *Phaeomoniella chlamydospora* in samples collected from nurseries at different propagation stages

Stage of grapevine propagation process	No. of samples[b]	Number of positive samples [a]			
		Nested-PCR		Culturing on medium	
		Phaeo[c]	Pch[d]	Phaeo	Pch
Hydration tanks	30	2	0	0	0
Scissors	28	0	0	1 (Pal[e]), 1 (Ppa[f])	0
Omega-cut grafting machines	13	1	1	0	0
Peat for root development	18	6	0	0	1

[a] Detection was performed by both nested PCR and filtration and incubation on semi-selective culture medium

[b] Number of samples analysed

[c] *Phaeoacremonium* spp.

[d] *Phaeomoniella chlamydospora*

[e] *Phaeoacremonium aleophilum*

[f] *Phaeoacremonium parasiticum*

Table 2 Frequency and percentage of fungal trunk pathogens detected in grapevine rootstock mother fields sampled in Spain in 2006 and 2007[a]

	June 06		Nov 06		June 07		Nov 07	
	No. Pl	%[b]	No. Pl	%	No. Pl	%	No. Pl	%
Phaeoacremonium aleophilum	3	2.1	–	–	2	1.4	–	–
Phaeoacremonium parasiticum	–	–	1	0.7	–	–	–	–
Phaeoamoniella chlamydospora	–	–	4	2.9	–	–	–	–
Diplodia seriata	1	0.7	7	5	4	2.9	22	15.7
Neofusicoccum parvum	–	–	5	3.6	1	0.7	14	10
Botryosphaeria dothidea	1	0.7	2	1.4	–	–	1	0.7
Lasiodiplodia theobromae	–	–	1	0.7	–	–	1	0.7
Neofusicoccum australe	–	–	–	–	–	–	1	0.7
Neofusicoccum mediterraneum	–	–	–	–	–	–	1	0.7
Neofusicoccum vitifusiforme	–	–	–	–	–	–	1	0.7
Total Botryosphaeriaceae	2	1.4	15	10.7	5	3.6	40	28.5
Phomopsis viticola	–	–	–	–	2	1.4	–	–
Phomopsis sp1	–	–	–	–	1	0.7	–	–
Diaporthe helianthi	1	0.7	–	–	–	–	–	–
Total *Phomopsis* spp.	1	0.7	–	–	3	2.1	–	–
Total infected plants [c]	6	4.3	18	13.6	10	7.1	40	28.5

[a] Seven different fields were analysed each year; same plants (20 plants per field) were sampled in June and November for each field

[b] Percentage of infected plants over the total of 140 plants analysed in each survey (seven fields and 20 plants per field)

[c] Some plants were infected with several fungal trunk pathogens

any sampled plant in November 2006 although the same plants were sampled in June and November (Table 2). All pathogens were found in fields of 110 R rootstock (Table 3).

In 2007, there were ten plants infected in the first survey done in June (7.1%), while in November, 40 plants were infected (28.5%). In June 2007, *Pm. aleophilum*, Botryosphaeriaceae isolates and *Phomopsis* spp. (*Phomopsis viticola* and *Phomopsis* sp1) were respectively isolated from 1.4%, 3.6% and 2.1% of the plants. In November 2007, Botryosphaeriaceae isolates were obtained from 28.5% of the plants. One of these

Table 3 Number of plants infected with fungal trunk pathogens in grapevine rootstock mother fields sampled in November and June, in 2006 and 2007[a]

	Grapevine mother fields [b]																				
	2	3		4		5	6		7	8		9	10		11		12		13	14	
Species [c]	N	J	N	J	N	N	J	N	N	J	N	N	J	N	J	N	J	N	N	J	N
Pal	– [d]	1	–	–	–	–	1	–	–	–	–	–	–	–	1	–	1	–	–	–	–
Ppa	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Pch	–	–	–	–	–	1	–	1	–	–	–	–	–	–	–	–	–	–	–	–	–
Botry	1	1	4	1	4	3	–	4	1	2	7	4	1	7	1	6	1	6	4	–	6
Phomp.	–	1	–	–	–	1	–	–	–	–	–	–	1	–	–	–	–	–	–	2	–
Total[e]	1	7		4		4	6		1	7		4	8		6		6		4	7	

[a] Fields sampled in 2006 (1–7) and 2007 (8–14). Same plants were sampled in June (J) and November (N) for each field. Only samplings where pathogens were detected are shown

[b] Grapevine mother fields were 110 R rootstock, with the exception of field 1(140 Ru), field 13 (SO4) and field 14 (1103-Pa)

[c] Species detected: Pal: *Phaeoacremonium aleophilum*; Ppa: *P. parasiticum*; Pch: *Phaeomoniella chlamydospora*; Botry: Botryosphaeriaceae; Phomp: *Phomopsis* spp.

[d] No pathogens detected

[e] Some plants were infected with several trunk disease pathogens

plants was simultaneously infected by *Neofusicoccum mediterraneum* and *Diplodia seriata*. *Phaeoacremonium aleophilum* was not isolated from any sampled plant in November 2007 although the same plants were sampled in June and November (Table 2). A total of 73.8% of infected plants were 110 R rootstock, 16.7% 1103 P and 9.5% SO4. In this year, none of the fields were free of pathogens (Table 3).

We identified the following species among Botryosphaeriaceae isolates: *D. seriata*, *Botryosphaeria dothidea*, *Lasiodiplodia theobromae*, *Neofusicoccum parvum*, *N. australe*, *N. mediterraneum* and *N. vitifusiforme* (Table 2). As far as we know, *N. australe*, *N. mediterraneum* and *N. vitifusiforme* had not been previously reported occurring in grapevines in Spain, and *L. theobromae* was recently reported (Aroca et al. 2008b). Moreover, to our knowledge, this is the first report on the occurrence of *N. mediterraneum* on grapevine. These pathogens were isolated from both asymptomatic and symptomatic plants which showed chlorotic and small leaves or dead shoots.

Discussion

This work confirms that the propagation process of grapevine plants should be considered as an important source of inoculum for Petri disease pathogens (*Pa. chlamydospora* and *Phaeoacremonium* spp.). Previous work focused mostly on the detection of *Pa. chlamydospora* in nurseries and rootstock canes (Ridgway et al. 2002; Retief et al. 2006; Whiteman et al. 2004, 2007; Pollastro et al. 2009; Edwards et al. 2007) and the presence of *Phaeoacremonium* spp. was determined only in rootstock mothervines from South Africa (Fourie and Halleen 2002). Here we demonstrate that the propagation process in nurseries is also a potential source of inoculum for *Phaeoacremonium* spp. We detected Petri disease pathogens in hydration tanks, omega-cut grafting machines, scissors and callusing peat. No pathogens were detected in tap water and peat before being used at their respective stages, which indicates that they were present in water and peat after being in contact with infected planting material. We also demonstrated that viable propagules are present at different stages of the grafted plant production process, since *Pm. aleophilum* and *Pm. parasiticum* were isolated from scissors washings and *Pa. chlamydospora* from a peat sample using the semi-selective culture medium.

Grapevine rootstock mother plants are also an important inoculum source for Petri disease and other fungal trunk pathogens in the propagation process in Spain. Most of the sampled fields were of 110 R rootstock. This is one of the rootstocks more commonly planted in Spain because of its vigour, resistance to drought and good adaptation to the Spanish warm climate (Hidalgo 2002). Petri disease pathogens such as *Pa. chlamydospora*, *Pm. aleophilum*, *Pm. parasiticum*, several Botryosphaeriaceae species and *Phomopsis viticola* were isolated from the sampled rootstock mother vines. These results agree with those obtained from surveys of grapevine rootstock mother plants in other grapevine-growing areas worldwide (Fourie and Halleen 2002, 2004a; Ridgway et al. 2002; Whiteman et al. 2004, 2007). All these species had been previously found in diseased young vineyards surveyed in Spain (Armengol et al. 2001; Aroca et al. 2006, 2008b), with the exception of *N. australe, N. mediterraneum* and *N. vitifusiforme*, which are reported here for the first time in Spain. The small number of rootstock mother fields sampled in this work does not allow a statistical comparison, but the frequency of *Pa. chlamydospora* infection seems to be relatively low (2.9%) compared with that found in grapevine mother fields in South Africa and New Zealand, which were 25 and 18% respectively (Whiteman et al. 2004; Retief et al. 2006). Nevertheless, frequency of *Phaeoacremonium* spp. infection is relatively high (1.4%) in comparison with that found in South Africa (0.12%) (Fourie and Halleen 2004a). Other pathogen frequencies, *Phomopsis* spp. (1.4%) and Botryosphaeriaceae (5.2%), are not noticeably different from those found in other countries (Fourie and Halleen 2004a).

Fungal species isolated in the same plants from rootstock mother fields were different in June and November. Pathogens found in grapevine mother plants in June were *Pm. aleophilum*, *Phomopsis viticola* and some Botryosphaeriaceae spp. while the pathogens isolated more frequently in November were Botryospaheriaceae spp. and *Pa. chlamydospora*. This result suggests the need to establish a proper calendar to survey mother plant fields for specific pathogens within a certification programme. Furthermore, these surveys should be exhaustive since the presence of pathogens in the grapevine rootstock

mother plants were not related with the observed external symptoms. These results were also observed in surveys carried out in other countries (Halleen et al. 2003; Fourie and Halleen 2004a) and demonstrate the high potential risk of mother vines as an inoculum source in the vegetative propagation process.

Pathogens detected by nested PCR were different from those detected by culturing on a growth medium. Sensitivity of the detection method by PCR is higher than by plating on a growth medium (Aroca and Raposo 2007), and so a greater number of positive samples for *Phaeoacremonium* spp. was detected, although two samples from scissors were positive using the culturing method but not by PCR; *Pa. chlamydospora* was detected in different samples. Variation in results when the level of the pathogen is low has been observed when using PCR to detect *Pa. chlamydospora* (Edwards et al. 2007), possibly due to the fact that the pathogen is present at the limit of detection. This may be occurring here, indicating the need for a more intensive sub-sampling of each of the samples, in order to improve the reproducibility of detection. This would be mandatory for a quantitative study, but the scope of the work presented here is only qualitative and the presence of any pathogen is enough to show the potential risk of infection of the plant material.

Another possible inoculum source for Petri disease pathogens not studied here is the soil in the field where grafting plants are planted once the grafting propagation process is over. During this period of time grafted plants will start to grow, producing new roots that may be infected, as Whiteman et al. (2004) demonstrated for plants growing in artificially infected soil with *Pa. chlamydospora*. Although soil from nursery fields was not sampled in this work, a previous survey performed in Spain suggests that plants may also be infected during this stage. Giménez-Jaime et al. (2006) surveyed cuttings at different stages of the grafting process, and *Pm. aleophilum* and *Pa. chlamydospora* were exclusively isolated from grafted plants two months after they were planted in the nursery field. Another reason pointed out was that pathogens may require a higher level of host colonisation to be detected by isolation on culture media. If so, it is important to have a reliable and sensitive method for detecting Petri disease pathogens, since these fungi are not easily isolated by conventional methods.

Results presented in this work demonstrate that planting material used for grapevine propagation in Spain is infected with fungal trunk pathogens and that healthy cuttings may also be infected by *Pa. chlamydospora* and *Phaeoacremonium* spp. during the propagation process in nurseries. In this context, a sanitation programme is required to improve the quality of grapevine planting material. On the basis of recent research (Fourie and Halleen 2004b; Gramaje et al. 2009b, 2009c), various proactive management strategies have been recommended for the prevention of infection of propagation material by trunk disease pathogens during the grapevine nursery stages.

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