Phytopathol. Mediterr. (2011) 50 (Supplement), S77–S85

Phaeomoniella chlamydospora and *Phaeoacremonium* spp. in grapevines from Uruguay

EDUARDO ABREO, SEBASTIÁN MARTÍNEZ, LINA BETTUCCI and SANDRA LUPO

Laboratorio de Micología, Facultad de Ciencias, Facultad de Ingeniería, Julio Herrera y Reissig 565, C.P. 11300 Montevideo, Uruguay

Summary. Symptoms corresponding to esca and Petri diseases have been described in Uruguay as being associated with *Phaeomoniella chlamydospora* and *Phaeoacremonium* spp. Isolates of *Phaeoacremonium* spp. recovered from diseased grapevines were characterized and identified. Additionally, specific primers developed for *Pa. chlamydospora* and *Phaeoacremonium* spp. were evaluated for direct detection of these fungi in asymptomatic grapevine tissues. The bark was removed, and the trunk underneath was surface-disinfected. Chips from symptomatic grapevines were plated on potato dextrose agar and incubated at 25°C. Isolates were identified morphologically and phylogenetically. Sequences of ITS rDNA, β-tubulin and actin genes made it possible to identify 52 isolates as *Pa. chlamydospora*, 33 as *Pm. aleophilum,* and one as *Pm. australiense.* The *Pm. aleophilum* isolates were divided into three groups by their growth pattern and their colony shape at 37°C: a) white colonies with yellow or brown reverse; b) brown colonies with clear margin and a dark center on the reverse; c) brown colonies with dark-brown diffusible pigments and brown reverse. Further studies are required to explain these differences. Primers Pac1f/Pac2r specific for *Phaeoacremonium* spp., Pal1N/Pal2 specific for *Pm. aleophilum*, F2bt/R1bt specific for *Pm. aleophilum* and ten other *Phaeoacremonium* species, and Pch1/Pch2 and Pmo1f/Pmo2r, both specific for *Pa. chlamydospora* were evaluated on the DNA of target fungi and some of other fungi frequently isolated from diseased vine tissues. F2bt/R1bt and Pmo1f/ Pmo2r were selected, and were used in a nested PCR to detect *Phaeoacremonium* spp. and *Pa. chlamydospora* in asymptomatic canes of nursery mother grapevines. Nine out of ten sampled grapevines tested positive for one of the fungi. Molecular diagnosis is potentially a useful method to assess the health of mother grapevines.

Key words: Petri disease, esca, asymptomatic canes, monitoring, specific primers.

Introduction

In Uruguay, 8000 ha are used for viticulture, and several cultivars of *Vitis vinifera* are grown. Symptoms corresponding to esca and Petri disease have been seen in up to 4.3% of vines, with the incidence depending on the cultivar (Abreo *et al*., 2008). However, only *Phomopsis viticola* (Sacc.) Sacc. and *Phaeomoniella (Pa.) chlamydospora* (W. Gams, Crous, M. J. Wingf. & L. Mugnai) Crous & W. Gams have been recorded in Uruguay as pathogens associated with these symptoms (Koch *et al.,* 1981; Marroni and Abreo, 2005).

Corresponding author: E. Abreo
Fax: +598 27120626
E-mail: eabreo@fing.edu.uy

Petri disease is a decline of young grapevine plants caused by *Pa. chlamydospora* and *Phaeoacremonium* spp., in which black streaking can be seen in longitudinal cuts of grapevine trunks. *Pa. chlamydospora* is considered more virulent than *Phaeoacremonium aleophilum* W. Gams, Crous, M. J. Wingf. & L. Mugnai, but both these fungi cause internal necrotic lesions and cankers in mechanical inoculations (Laveau *et al*., 2009).

Esca causes variable symptoms that usually include interveinal chlorosis and necrosis of the leaves (Larignon and Dubois, 1997; Mugnai *et al*., 1999), berries with black spots, and in older vines wood deterioration of the trunk. Black spots are seen when the main trunks or arms of grapevines are cut transversely: these spots correspond to black streaks when the trunks are cut lengthwise (Mugnai *et al*., 1999). Esca proper differs from in-

fections in young vines by also having white rot of the wood, caused by basidiomycete fungi (Mugnai *et al*., 1999; Surico *et al*., 2006; Surico, 2009). These basidiomycete fungi belong to *Fomitiporia* spp. in the northern hemisphere (Fischer, 2006), but in Argentina and Uruguay the species principally associated with white rot of *Vitis vinifera* is *Inocutis jamaicensis* (Murrill) Gottlieb, J.E. Wright & Moncalvo (Lupo *et al*., 2006; Perez *et al.,* 2008).

The genus *Phaeoacremonium* W. Gams, Crous & M. J. Wingf. (Crous *et al*., 1996), currently contains more than 20 species, of which about 15 species have been reported from *Vitis vinifera* growing in various parts of the world (Mostert *et al*., 2003, 2006a; Damm *et al*., 2008; Essakhi *et al*., 2008; Gramaje *et al*., 2009). The taxon *Phaeomoniella* was established to accommodate *Phaeoacremonium chlamydosporum*, which showed to be unrelated to the genus *Phaeoacremonium* (Crous and Gams, 2000).

Traditional microbiological methods of identification are difficult and time-consuming, and can produce false negative results when infection levels are low. Molecular tools based on the sequence of partial β-tubulin and actin genes have been used to identify a number of *Phaeoacremonium* species (Mostert *et al*. 2006b; Aroca *et al*. 2008; Graham *et al*. 2009), whereas specific primers from the ITS region are used to detect *Pa. chlamydospora* (Tegli *et al.,* 2000; Overton *et al*., 2004).

The early diagnosis of infected asymptomatic plants is based on the detection of fungal DNA by PCR. Specific primers are an effective and sensitive means to detect *Pa. chlamydospora* and *Phaeoacremonium* spp. in infected wood (Tegli *et al*., 2000; Ridgway *et al*., 2002; Overton *et al.,* 2004). Whiteman *et al*. (2002) used nested PCR to detect *Pa. chlamydospora* in the soil. Similarly, Aroca *et al*. (2008) designed a degenerate primer pair (F2bt–R1bt) with homology to the β-tubulin gene to amplify 11 species of *Phaeoacremonium* aiming at early detection.

The main goal of the present work was to determine the occurrence of *Pa. chlamydospora* and species of *Phaeoacremonium* associated with esca and Petri disease of grapevine in Uruguay. In addition, previously developed PCR-based diagnostic tests were evaluated for their applicability in asymptomatic canes from nurseries in Uruguay.

Materials and methods

Fungal isolates

One hundred grapevines showing external decline symptoms and 37 externally asymptomatic plantlets of age less than 2 years from 30 vineyards located in the grape-growing regions of Uruguay were collected. The bark was removed from the trunk samples, and the cleared trunk of each sample was surface-disinfected. Chips were cut from the inner tissues, and incubated on potato dextrose agar medium (PDA, Difco, Sparks, MD, USA) at 25°C. Fungal isolates were identified to genus level by macro- and micromorphological characteristics.

Cultural characteristics

Twenty-six isolates were subjected to morphological examination (Table 1). Growth characteristics were determined on 90 mm Petri dishes containing malt extract agar (MEA; 2% malt extract, 1.5 % agar, Difco). The growth rate of the colonies at 25°C was measured after 1 and 2 weeks. Colony characteristics were studied after 4 weeks, including color and shape, center, border, aerial mycelia, and production of yellow pigment (Gams *et al.,* 2007). Growth and changes in shape on MEA (Difco) at 37°C was also recorded.

DNA isolation and amplification from mycelium

Twenty-eight isolates of *Phaeoacremonium* spp. from different localities and substrates were selected for DNA analysis (Table 1). Colonies for DNA extraction were grown on Petri dishes containing PDA until colony size reached a diameter of 20 mm, which required 10 to 15 days depending on the isolate. Mycelium was harvested with a sterile scalpel and the DNA was extracted and purified according to the protocol of Lee and Taylor (1990). PCR amplification of the partial β-tubulin gene was performed using primers T1/Bt2b (Glass and Donaldson, 1995; O'Donnell and Cigelnik, 1997). The actin gene sequences of one representative strain of each morphotype (strain and GenBank accession numbers: FI2103, HQ159871; FI2105, HQ159872; FI2106, HQ159873) were amplified with primers ACT-512F/ACT-783R (Carbone and Kohn, 1999). The amplified segments were visualized under UV lighting in agarose gel (1%), and purified and sequenced by Macrogen (Seoul, Korea).

Phylogenetic analysis

The sequences obtained were analyzed in MEGA4 (Tamura *et al*., 2007) and aligned using ClustalW; additional reference sequences were obtained from GenBank and added to the alignments (Table 1). Aligned sequences of the β-tubulin gene, alone and concatenated with the sequences of the actin gene, were subjected to phylogenetic analysis using parsimony (PAUP; v. 4.0b10; Swofford, 2003). All characters were treated as unordered and of equal weight, and gaps were treated as missing data. Maximum parsimony analysis was performed using the heuristic search option with 100 random simple taxa additions and tree bisection and reconstruction (TBR) as the branch swapping algorithm. Branches of zero length were collapsed and 1000 multiple, equally parsimonious trees were saved. The robustness of the trees obtained was evaluated by 1000 bootstrap replications (Hillis and Bull, 1993). The tree length (TL), consistency index (CI), retention index (RI) and rescaled consistency index (RC) were calculated. The new sequences obtained are deposited in GenBank. *Pleurostomophora richardsiae* (CBS 270.33, GenBank AY579334) was used as outgroup in the phylogenetic analysis (Damm *et al*., 2008; Essakhi *et al*., 2008).

Primer specificity

The specificity of the published primers for *Phaeoacremonium* spp. and *Pa. chlamydospora* was evaluated on target DNA from the Uruguayan isolates, and a set of DNA from some fungi found in close association with the target species in symptomatic tissues. The specific primers assessed were: Pac1f/Pac2r for *Phaeoacremonium* spp., Pal1N/Pal2 for *Pm. aleophilum*, and F2bt/R1bt for *Pm. aleophilum* and ten other *Phaeoacremonium* species from grapevine (Tegli *et al.,* 2000; Overton *et al.,* 2004; Aroca *et al*., 2008). The specific primers Pch1/Pch2 and Pmo1f/Pmo2r were evaluated for *Pa. chlamydospora* (Tegli *et al*., 2000; Overton *et al*., 2004).

Detection of *Phaeoacremonium* spp. and *Pa. chlamydospora* in grapevine wood

Primers that were effective and specific were further assessed for their capacity to directly amplify target DNA from DNA isolated from asymptomatic canes of mother grapevines in nurseries.

One cane each of five vines cv. Tannat and rootstock '3309C' was sampled. Each cane was cut into basal, middle and apical segments. A total of 30 segments were analyzed. The segments were stripped of their bark and surface-disinfected. Small fragments of xylem wood were cut with a sterile scalpel and the DNA from these fragments was purified using the AxyPrep Multisource Genomic DNA Miniprep Kit (Axygen, Union City, CA, USA). The DNA was amplified by nested PCR with universal primers ITS4/ITS5 and T1/Bt2b, and specific primers Pmo1F/Pmo2R for *Pa. chlamydospora* and F2Bt/R1bt for *Phaeoacremonium* spp.

Results

Thirty-two isolates of *Phaeoacremonium* and 51 of *Pa. chlamydospora* were obtained from symptomatic grapevines, and only two isolates of *Phaeoacremonium* and one of *Pa. chlamydospora* from asymptomatic plantlets. Thirty-three isolates were assigned to *Pm. aleophilum* and one to *Pm. australiense* by their morphological characteristics and phylogenetic analysis. *Pm. aleophilum* was isolated in all wine-growing regions in Uruguay from the cultivars Tannat, Cabernet Franc, Cabernet Sauvignon, Shiraz, Gewürztraminer and from rootstocks 3309C and SO4. Symptoms included a general decline of young grapevines, dead arm, and the decline and collapse of older vines. Only one isolate of *Pm. australiense* was obtained; it came from a declining Cabernet Sauvignon grapevine from Colonia in the southwest of the country (Table 1). Both species are recorded for the first time in Uruguay. *Pa. chlamydospora* was isolated from the same cultivars, and more frequently than *Phaeoacremonium* species. The higher ratio of *Pa. chlamydospora* was found at all sampling sites.

Cultural characteristics

Growth and colony shape were similar for all isolates of *Pm. aleophilum* grown at 25ºC. However, at 37°C on PDA these characteristics differed (Figure 1) and in these conditions isolates could be grouped into three morphotypes producing: a) short white woolly to cottony colonies, with wet and whitish margin and yellowish or brownish pigment in the reverse and under the inoculum; b) woolly to felty colonies, yellowish to reddish brown

around the inoculum, with entire white margins and brown reverse; c) ceraceous to crustose, greyish brown colonies, felty around the inoculum and with diffuse margins, also greyish brown, and with brown pigments diffusing into the medium under and around the colony margin.

Phylogenetic analysis

A selection of 28 Uruguayan isolates of *Phaeoacremonium* was used in the phylogenetic analysis based on the partial β-tubulin gene along with 29 sequences from GenBank (Table 1). After alignment the final data set contained 599 characters including the gaps, of which 274 were parsimony-informative, 88 were variable and parsimony-uninformative and 237 were constant. Parsimony analysis yielded 90 equally most parsimonious trees with the same overall topology differing mainly in the order of the taxa at the terminal nodes. The 50% majority rule consensus tree is shown in Figure 2 (TL = 1045; CI = 0.5933; RI = 0.8365; RC = 0.4963; HI = 0.4541). Based on the DNA sequences, two species of *Phaeoacremonium* were identified. Twenty seven isolates clustered with *Pm. aleophilum* (100% bootstrap support) and one isolate clustered with a clade containing *Pm. australiense* isolates retrieved from GenBank (100% bootstrap support), including ex-type strains for both species (Figure 2). Uruguayan isolates shared 13 sequence variations in comparison with the *Pm. aleophilum* ex-type strain, including a four base deletion in position 50–53. Other variations in the sequences of Uruguayan isolates did not reflect the differences among the morphotypes (Table 1).

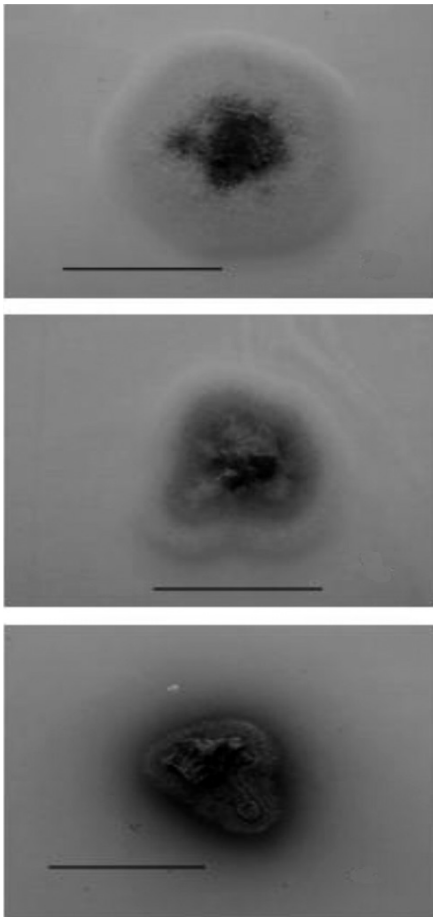

Figure 1. Colony morphology of *Phaeoacremonium aleophilum* groups (a,b,c) grown on malt extract agar at 37°C for 14 days (scale bar=10 mm)

Concatenated phylogenetic analysis of the β-tubulin and actin partial sequences clustered the different morphotypes with the type strain sequences of *Pm. aleophilum*, indicating that they all belonged to the same species (data not shown).

Primer specificity

The primer pair Pal1N/Pal2 did not amplify the DNA of *Pm. aleophilum* in the PCR conditions. Pch1/Pch2 did not retrieve *Pa. chlamydospora* in the PCR conditions. Pac1f/Pac2r produced amplicons of the expected size, but in some cases, when tested on DNA extracted from grapevine wood, their sequences did not belong to the genus *Phaeoacremonium* (90% homology with *Chaetosphaeria tulasneorum* AF178547*)*. Pmo1f/Pmo2r and F2bt/R1bt amplified the local isolates of *Pa. chlamydospora* and *Pm. aleophilum*, respectively (Table 2).

Detection of *Phaeoacremonium* spp. and *Pa. chlamydospora* in asymptomatic canes with specific primers

The primers Pmo1f/Pmo2r and F2Bt/R1Bt used to amplify target DNA from segments of healthy canes produced positive results: nine out of ten canes and grapevines were positive for at least one target fungus. Of the 30 segments obtained from these canes, 13 resulted positive for *Pa. chlamydospora*, and six for *Phaeoacremonium* spp. (Table 3). Analyses of sequences indicated high homology of the latter with *Pm. aleophilum* (data not shown).

Table 1. Isolates, accession numbers for β-tubulin sequences and location, host and symptom details for Uruguayan *Phaeoacremonium* spp. included in phylogenetic and morphological studies. Isolates numbers in bold were sequenced for the present study.

Isolates	Taxon	Location	Host	Symptoms	Genbank acc. No.
FI 2084	*Phaeoacremonium aleophilum*	Canelones	*V. vinifera* cv. Cabernet Franc	Decline	HQ159844
FI 2085	*Pm. aleophilum*	Canelones	*V. vinifera* cv. Cabernet Franc	Decline	HQ159845
FI 2086	*Pm. aleophilum*[3]	Canelones	*V. vinifera* cv. Gewurztraminer	Old vine decline	HQ159846
FI 2087	*Pm. aleophilum*	Canelones	Root stock unknown	Old vine decline	HQ159847
FI 2088	*Pm. aleophilum*	Canelones	Root stock unknown	Old vine decline	HQ159848
FI 2089	*Pm. aleophilum*[1]	Colonia	Root stock 3309C	Dead vine	HQ159849
FI 2112	*Pm. aleophilum*[3]	Colonia	Root stock 3309C	Decline	
FI 2090	*Pm. aleophilum*[3]	Colonia	Root stock 3309C	Decline	HQ159850
FI 2113	*Pm. aleophilum*[1]	Colonia	Root stock 3309C	Decline	
FI 2111	*Pm. australiense*	Colonia	*V. vinifera* cv. Cabernet Sauvignon	Decline	HQ159870
FI 2091	*Pm. aleophilum*[3]	Colonia	*V. vinifera* cv. Syrah	Dead arm	HQ159851
FI V105	*Pm. aleophilum*[3]	Colonia	*V. vinifera* cv. Syrah	Dead arm	
FI 2092	*Pm. aleophilum*[1]	Colonia	*V. vinifera* cv. Cabernet Sauvignon	Old vine decline	HQ159852
FI 2114	*Pm. aleophilum*[2]	Paysandú	*V. vinifera* cv. Moscatel Otonel	Dead arm	
FI 2115	*Pm. aleophilum*[3]	Artigas	Root stock unknown	Decline	
FI 2094	*Pm. aleophilum*[3]	Artigas	Root stock unknown	Decline	HQ159854
FI 2095	*Pm. aleophilum*[3]	Artigas	*V. vinifera* cv. Cabernet Sauvignon	Dead arm	HQ159855
FI 2096	*Pm. aleophilum*[3]	Artigas	Root stock unknown	Decline	HQ159856
FI 2097	*Pm. aleophilum*[3]	Artigas	Root stock unknown	Decline	HQ159857
FI 2098	*Pm. aleophilum*[3]	Paysandú	*V. vinifera* cv.Cabernet Sauvignon	Dead arm	HQ159858
FI 2099	*Pm. aleophilum*[3]	Paysandú	*V. vinifera* cv.Tannat	Dead arm	HQ159859
FI 2100	*Pm. aleophilum*[3]	Paysandú	*V. vinifera* cv.Tannat	Dead arm	HQ159869
FI 2101	*Pm. aleophilum*[3]	Paysandú	*V. vinifera* cv.Tannat	Dead arm	HQ159860
FI 2102	*Pm. aleophilum*[3]	Salto	Root stock unknown	Decline	HQ159861
FI 2116	*Pm. aleophilum*[3]	Salto	Root stock unknown	Decline	
FI 2103	*Pm. aleophilum*[2]	Canelones	*V. vinifera* cv.Cabernet Sauvignon	Unknown	HQ159862
FI 2104	*Pm. aleophilum*	Artigas	Root stock unknown	Decline	HQ159863
FI 2105	*Pm. aleophilum*[3]	Tacuarembó	Cv. unknown	Dead arm	HQ159864
FI 2106	*Pm. aleophilum*[1]	Rivera	Cv. unknown	Dead arm	HQ159865
FI 2107	*Pm. aleophilum*[1]	Rivera	Root stock unknown	Old vine decline	HQ159866
FI 2093	*Pm. aleophilum*[1]	Rivera	Cv. unknown	Unknown	HQ159853
FI 2108	*Pm. aleophilum*	Maldonado	Root stock unknown	Decline	HQ159867
FI 2109	*Pm. aleophilum*	Canelones	*V. vinifera* cv. Tannat	Asymptomatic plantlet	HQ159868
FI 2110	*P. aleophilum*	Canelones	*V. vinifera* cv. Cabernet Franc	Asymptomatic plantlet	HQ159843

[1, 2, 3] Morphotypes a, b and c respectively.

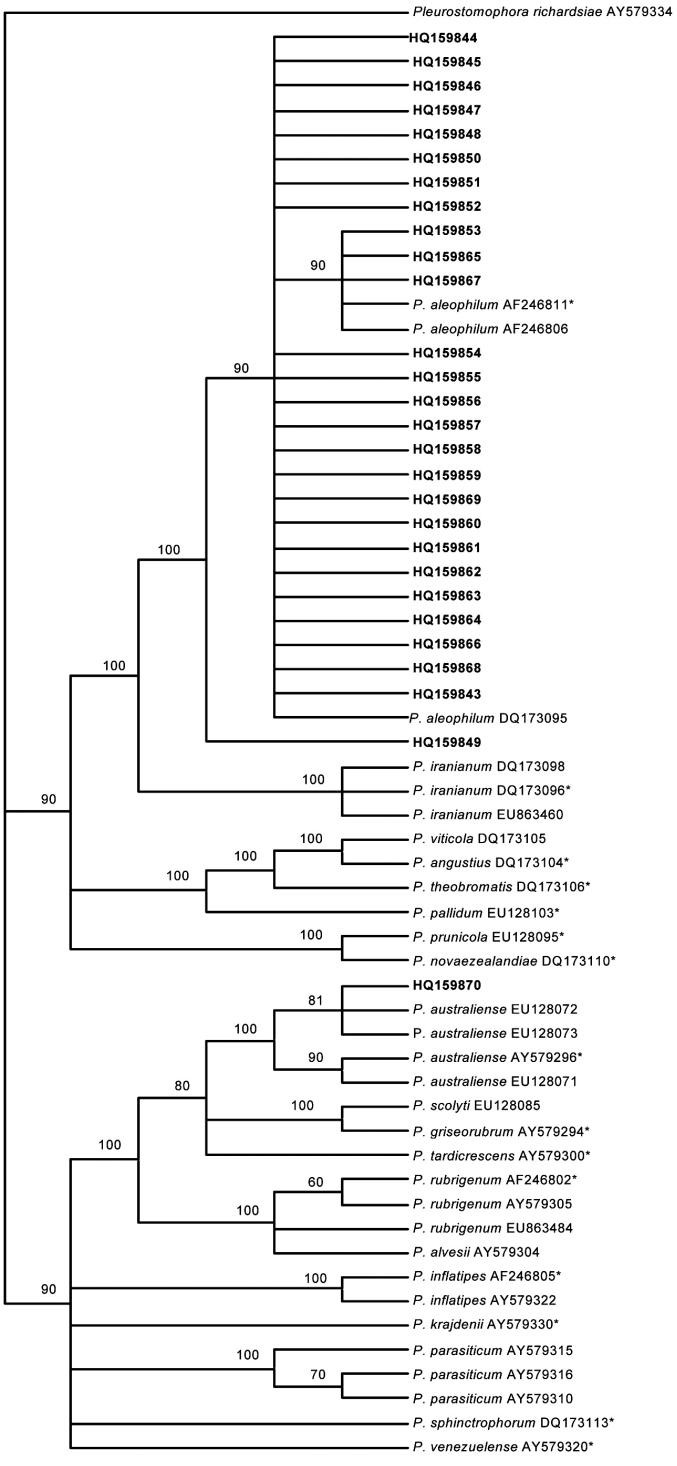

Figure 2. Fifty percent majority rule consensus phylogenetic tree obtained using β-tubulin sequences of *Phaeoacremonium* species obtained in the present study and from GenBank. GenBank numbers in bold were sequenced for the present analysis. Sequences from ex-type cultures are indicated by *. Bootstrap support values based on 1000 replicates are shown at the nodes.

Table 2. PCR amplification of DNA from selected fungal species isolated from grapevines with specific primers for *Phaeoacremonium* spp. and *Phaeomoniella chlamydospora*.

Fungal species	Primer[a]			
	Pac1f/Pac2r	F2Bt/R1Bt	Pmo1f/Pmo2r	ITS4/ITS5[b]
Pm. aleophilum (36 isolates)	+	+	-	+
Pm. australiense	+	-	-	+
Pa. chlamydospora (11 isolates)	-	-	+	+
Inocutis jamaicensis	-	-	-	+
Botryosphaeria parva	-	-	-	+
Phomopsis viticola	-	-	-	+
Phomopsis sp. 1	-	-	-	+
Phomopsis sp. 2	-	-	-	+
Hymenochaetaceae	-	-	-	+
Greeneria uvicola	-	-	-	+
Altenaria alternata	-	-	-	+
Campylocarpon pseudofasciculare	-	-	-	+
Cadophora melinii	-	-	-	+
Eutypella vitis	-	-	-	+

[a] (+) Amplification, (-) no amplification.
[b] ITS region amplified as positive control.

Discussion

Almost twice as many isolates of *Pa. chlamydospora* than *Phaeoacremonium* spp. were obtained when symptomatic plants from established vineyards were analyzed. Zanzotto *et al*. (2007) found that even though *Phaeoacremonium* species were more prevalent within the fungal population isolated from rooted grafts, their incidence decreased in four-year-old grapevines, whereas *Pa. chlamydospora* slowly increased. Larignon and Dubos (2000) found that spores of *Pa. chlamydospora,* produced under field conditions, infected trunks and canes through pruning wounds in winter. *Phaeoacremonium* spp. did not do so since they did not produce airborne spores in winter. In California, Eskalen & Gubler (2001) did find that *Pm. aleophilum* produced spores in winter, but the frequencies were lower than in summer. These findings indicate that infected nursery material is an important mean of spreading *Phaeoacremonium* spp., whereas the spread and prevalence of *Pa. chlamydospora* in established vineyards could be enhanced by the often more common aerial infec-

Table 3. PCR amplification of DNA from cuttings from different positions on different grapevine cultivars, using selected specific primers F2Bt/R1Bt for *Phaeoacremonium* spp. and Pmo1f/Pmo2r for *Phaeomoniella chlamydospora*.

Sample	Cv. Tannat[a]			Rootstock 3309C[a]		
	Basal	Medium	Apical	Basal	Medium	Apical
Cane 1	P	P		Pa	Pa	
Cane 2		Pa	P	Pa		Pa
Cane 3	P	P	P	Pa		
Cane 4	Pa	Pa		Pa		Pa
Cane 5				Pa	Pa	Pa

[a] Pa, amplification of *Phaeomoniella chlamydospora*; P, amplification of *Phaeoacremonium aleophilum*.

tion by this fungus through pruning wounds. This would explain the higher frequency of *Pa. chlamydospora* under field conditions.

Primers Pal1N/Pal2 did not amplify target DNA from pure cultures under the conditions specified by the designers and were excluded from further tests to detect target DNA in asymptomatic canes. It should be pointed out that Romanazzi *et al*. (2009) using these primers did not detect *Pm. aleophilum* in asymptomatic tissues, but they did isolate it on artificial growth medium.

Primers Pac1r/Pac2f were specific in the initial specificity trial on pure fungal DNA. However, when used on DNA from grapevine wood, even though they generated PCR products of the correct size, the sequences of these products belonged to non-target species. On the other hand, the specific primers F2bt/R1bt with homology to the β-tubulin gene detected *Pm. aleophilum* in a nested PCR reaction in healthy-looking canes, as confirmed by sequence analysis, while they did not amplify non target DNA.

Nested PCR with the selected primers indicated that eight out of ten basal cuttings were positive for *Pa. chlamydospora* or *Pm. aleophilum*, but this ratio diminished to six out of ten in medium cuttings, and five out of ten in apical cuttings, in an apparent trend towards lower contamination levels in the more distal parts of the grapevines. Similarly, Troccoli *et al*. (2001) found that colonization by *Pa. chlamydospora* inoculated in roots was greatest at root collar level, and became less frequent at the stem base to disappear altogether above the 7th and 8th internodes; this could suggest that internal contamination was slow to spread due to existing plant defense mechanisms.

The morphotypes of *Pm. aleophilum* growing at 37°C on PDA indicate that there are physiological differences within this species. Punctual variations among morphotypes in the sequence of the β-tubulin partial gene and the actin gene did not however separate any species other than *Pm. aleophilum*. The different morphotypes of *Pm. aleophilum* and their biology and pathogenicity requires further study.

The present study found that *Pa. chlamydospora* and *Pm. aleophilum* were almost the only species in established vineyards and nurseries in Uruguay, irrespective of the region, rootstock and cultivar. Their occurrence in cuttings from apparently healthy canes used in propagation, and the availability of sensitive primers to detect them, suggest molecular diagnosis should be regularly used to assess the health of mother grapevines.

Acknowledgements

The authors are grateful to INIA-FPTA, Agencia Nacional de Investigación e Innovación and PEDECIBA, Universidad de la República, Uruguay.

Literature cited

Abreo E., S. Lupo, S. Martinez and L. Bettucci, 2008. Fungal species associated to grapevine trunk diseases in Uruguay. *Journal of Plant Pathology* 90, 591.

Aroca A., R. Raposo and P. Lunello, 2008. A biomarker for the identification of four *Phaeoacremonium* species using the β-tubulin gene as the target sequence. *Applied Microbiology and Biotechnology* 80, 1131–1140.

Carbone I. and L.M. Kohn, 1999. A method for designing primer sets for speciation studies in filamentous ascomycetes. *Mycologia* 91, 553–556.

Crous P.W., W. Gams, M.J. Wingfield and P.S. Van Wyk, 1996. *Phaeoacremonium* gen. nov. associated with wilt and decline diseases of woody hosts and human infections. *Mycologia* 88, 786–796.

Crous P.W. and W. Gams, 2000. *Phaeomoniella chlamydospora* gen. et comb. nov., a causal organism of Petri grapevine decline and esca. *Phytopathologia Mediterranea* 39, 112–118.

Damm U., L. Mostert, P.W. Crous and, P.H. Fourie, 2008. Novel *Phaeoacremonium* species associated with necrotic wood of *Prunus* trees. *Persoonia* 20, 87–102.

Eskalen A. and W.D. Gubler, 2001. Association of spores of *Phaeomoniella chlamydospora*, *Phaeoacremonium inflatipes*, and *Pm. aleophilum* with grapevine cordons in California. *Phytopathologia Mediterranea* 40, S429-432.

Essakhi S., L. Mugnai, P.W. Crous, J.Z. Groenewald and G. Surico, 2008. Molecular and phenotypic characterisation of novel *Phaeoacremonium* species isolated from esca diseased grapevines. *Persoonia* 21, 119–134.

Fischer M., 2006. Biodiversity and geographic distribution of basidiomycetes causing esca-associated white rot in grapevine: a worldwide perspective. *Phytopathologia Mediterranea* 45, S30–S42.

Gams W., G.J.M. Verkley and P.W. Crous (ed.), 2007. *CBS Course of Mycology*. 5th edition. Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands, 165 pp.

Glass N.L. and G.C. Donaldson, 1995. Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. *Applied and Environmental Microbiology* 61, 1323–1330.

Graham A.B., P.R. Johnston and B.S. Weir, 2009. Three new *Phaeoacremonium* species on grapevines in New

Zealand. *Australasian Plant Pathology* 38, 1–9.
Gramaje D., J. Armengol, H. Mohammadi, Z. Banihashemi and L. Mostert, 2009. Novel *Phaeoacremonium* species associated with Petri disease and esca of grapevine in Iran. *Mycologia* 101, 920–929.
Hillis D.M. and J.J. Bull, 1993. An empirical test of bootstrapping as a method for assessing confidence in phylogenetic analysis. *Systematic Biology* 42, 182–192.
Koch L., C. Boasso, O. Riccio and C. Gandolfo (ed.), 1981. *Enfermedades de las Plantas, Hongos Superiores y Saprofitas en el Uruguay*. Departamento de Comunicaciones. Dirección de Sanidad Vegetal. M.A.P., Montevideo, Uruguay, 140 pp.
Larignon P. and B. Dubos, 1997. Fungi associated with esca disease in grapevine. *European Journal of Plant Pathology* 103, 147–157.
Larignon P. and B. Dubos, 2000. Preliminary studies on the biology of *Phaeoacremonium*. *Phytopathologia Mediterranea* 39, 184–189.
Laveau C., A. Letouze, G. Louvet, S. Bastien, and L. Guerin-Dubrana, 2009. Differential aggressiveness of fungi implicated in esca and associated diseases of grapevine in France. *Phytopathologia Mediterranea* 48, 32–46.
Lee S. and J. Taylor, 1990. Isolation of DNA from fungal mycelia and single spores. In: *PCR protocols: a guide to methods and applications*. (M.A. Innis, D.H. Gelfand, J.J. Snindky, T.J. White, ed.), Academic Press, San Diego, CA, USA, 282–287.
Lupo S., L. Bettucci, A. Pérez, S. Martínez, C. Césari, G. Escoriaza and M. Gatica, 2006. Characterization and identification of the basidiomycetous fungus associated to 'hoja de malvón' grapevine disease in Argentina. *Phytopathologia Mediterranea* 45, S110–S116.
Marroni M.V. and E. Abreo, 2005. *Phaeomoniella chlamydospora* associated with Petri disease in grapevines in Uruguay. In: *Thirteenth Biennial Australasian Plant Pathology Society Conference*, Geelong, Victoria, Australia, 136 (abstract).
Mostert L., P.W. Crous, J.Z. Groenewald, W. Gams and R. Summerbell, 2003. *Togninia* (Calosphaeriales) is confirmed as teleomorph of *Phaeoacremonium* by means of morphology, sexual compatibility, and DNA phylogeny. *Mycologia* 95, 646–659.
Mostert L., F. Halleen, P. Fourie and P.W.Crous, 2006a. A review of *Phaeoacremonium* species involved in Petri disease and esca of grapevines. *Phytopathologia Mediterranea* 45, S12–S29.
Mostert L., J.Z. Groenewald, R.C. Summerbell, W. Gams and P.W Crous, 2006b, Taxonomy and pathology of *Togninia* (*Diaporthales*) and its *Phaeoacremonium* anamorphs. *Studies in Mycology* 54, 1–115.
Mugnai L., A. Graniti and G. Surico, 1999. Esca (black measles) and brown wood-streaking: two old and elusive diseases of grapevines. *Plant Disease* 83, 404–418.
O`Donnell and E. Cigelnik, 1997. Two different intragenomics rDNA ITS2 types within a monophyletic lineage of the fungus *Fusarium* are nonorthologous. *Molecular Phylogenetics Evolution* 7, 103–116.
Overton B.E., E.L. Stewart, Q.U. Xinshun, N.G. Wenner and B.J. Christ, 2004. Qualitative real-time PCR SYBR Green detection of Petri disease fungi *Phytopathologia Mediterranea* 43, 403–410.
Peréz G., S. Lupo and L. Bettucci, 2008. Polymorphisms of the ITS region of *Inocutis jamaicensis* associated with *Eucalyptus globulus*, *Vitis vinifera* and native plants in Uruguay. *Sydowia* 60, 267–275.
Ridgway H., B. Sleight and A. Stewart, 2002. Molecular evidence for the presence of *Phaeomoniella chlamydospora* in New Zealand nurseries, and its detection in rootstocks mother vines using species-specific PCR. *Australasian Plant Pathology* 31, 267–271.
Romanazzi G., S. Murolo, L. Pizzichini and S. Nardi, 2009. Esca in young and mature vineyards, and molecular diagnosis of associated fungi. *European Journal of Plant Pathology* 125, 277–290.
Surico G., 2009. Towards a redefinition of the diseases within the esca complex of grapevine. *Phytopathologia Mediterranea* 48, 5–10.
Surico G., L. Mugnai and G. Marchi, 2006. Older and more recent observations on esca: a critical overview. *Phytopathologia Mediterranea* 45, S68–S86.
Swofford D.L., 2003. *PAUP. Phylogenetic Analysis Using Parsimony (and other methods)*. Version 4., Sinauer Associates, Sunderland, MA, USA.
Tamura K., J. Dudley, M. Nei and S. Kumar, 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Molecular Biology and Evolution* 24, 1596–1599.
Tegli S., E. Bertelli and G. Surico, 2000. Sequence analysis of ITS ribosomal DNA in five *Phaeoacremonium* species and development of a PCR-based assay for the detection of *P. chlamydospora* and *P. aleophilum* in grapevine tissue. *Phytopathologia Mediterranea* 39, 134–149.
Troccoli L., R. Calamassi, B. Mori, L. Mugnai and G. Surico, 2001. *Phaeomoniella chlamydospora*-grapevine interaction: histochemical reactions to fungal infection. *Phytopathologia Mediterranea* 40, S400–S406.
Whiteman S., M. Jaspers, A. Stewart and H. Ridgway, 2002. Detection of *Phaeomoniella chlamydospora* in soil using species-specific PCR. *New Zealand Plant Protection* 55, 139–145.
Zanzotto A., F. Autiero, D. Bellotto, G. Dal Cortivo, G. Lucchetta and M. Borgo, 2007. Occurrence of *Phaeoacremonium* spp. and *Phaeomoniella chlamydospora* in grape propagation materials and young grapevines. *European Journal of Plant Pathology* 119, 183–192.

Accepted for publication: February 15, 2010