

Fungal Pathogens Associated with Grapevine Trunk Diseases in Iran

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ABSTRACT

During 2004–2007 various own rooted vineyards were inspected to study the fungi associated with vine trunk diseases in Iran. Samples from declining vines showing yellowing and reduced growth and different symptoms in wood, including browning of the wood, necrosis, brown and black streaking and white rot were collected. Fungal isolations were made from affected tissues onto Malt Extract Agar (MEA) supplemented with 1 g l⁻¹ streptomycin sulphate (MEAS). Based on morphological and molecular characteristics, the following species were identified: *Phaeoacremonium* (*Pm.*) *aleophilum*, *Phaeomoniella* (*Pa.*) *chlamydospora* and less frequently *Pm. parasiticum*, *Pm. inflatipes*, *Pm. cinereum*, *Cylindrocarpon* *liriodendri*, *Diplodia seriata* and *Neofusicoccum parvum*. Results of the pathogenicity tests under field conditions showed that *Pa. chlamydospora* and *Phaeoacremonium* spp. caused large wood discoloration 10 months after inoculation without any external foliar symptoms. *Phaeomoniella chlamydospora* caused larger lesions than *Phaeoacremonium* spp. All inoculated species were re-isolated from the margin of the lesions completing Koch's postulates. This study represents the first comprehensive work that investigates the molecular and morphological identification and pathogenicity of *Phaeoacremonium* spp. and *Pa. chlamydospora* associated with vine decline in Iran. This is also the first report of *Pm. inflatipes*, *N. parvum* and *D. seriata* associated with grapevine decline in this country.

Keywords: Grapevine decline, *Phaeoacremonium*, *Phaeomoniella chlamydospora*

INTRODUCTION

Grapevine trunk diseases are some of the major limiting factors in grape production throughout the world. Several ascomycetes (eg *Eutypa lata* (Pers.:Fr.) Tul. & C. Tul., Botryosphaeriaceae species and *Cylindrocarpon* spp. Wollenw.), basidiomycetes (eg *Fomitiporia mediterranea* M. Fisch.) and such mitosporic fungi as *Phaeoacremonium aleophilum* W. Gams, Crous, M. J. Wingf. and L. Mugnai and

Phaeomoniella chlamydospora (W. Gams, Crous, M.J. Wingf. and L. Mugnai) (Crous and Gams, 2000) are known to cause trunk diseases in grapevine (Larignon and Dubos, 1997; Mugnai *et al.*, 1999). Esca and Petri disease are two of the most destructive diseases of grapevines worldwide. Esca is associated with mature grapevines, external symptoms being characterized by an interveinal chlorosis or reddening sing of the leaves known as “tiger stripes”, shoot tip dieback and gray to brown spots appearing on the berries (black measles) (Dubos and

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Larignon, 1988). Internal symptoms principally include black streaking of the xylem vessels, which sometimes can be associated with the presence of white rot that gradually transform the hard wood into soft-yellowish wood (Mugnai et al., 1999). Recently the term “esca” was restricted to grapevine wood rot and the term “Grapevine leaf stripe disease” used for tracheomycosis which is associated with tiger stripe symptoms on grapevine leaves (Surico, 2009). Fungi that have been associated with esca symptoms include the wood rotting basidiomycetes, *F. mediterranea* and to a lesser extent *Stereum hirsutum* (Willd.: Fr) Pers. as well as the hyphomycetes, *Pa. chlamydospora* and *Pm. aleophilum* (Larignon and Dubos, 1997; Mugnai et al., 1999; Ari, 2000). Petri disease causes stunted growth, shortened internodes, small leaves, reduced foliage, and brown to black spots or streaks in the xylem vessels of the vines (Adalat et al., 2000). Petri disease is caused by a combination of several fungi such as *Pa. chlamydospora* and different *Phaeoacremonium* species (Mugnai et al., 1999; Groenewald et al., 2001). Symptoms of Botryosphaeria canker consist of perennial cankers, trunk dieback, wood necrosis, wedge-shaped necrotic sectors, mild chlorosis and wilting of leaves (Castillo-Pando et al., 2001; Phillips, 2002; van Niekerk et al., 2004). Black foot disease, caused by *Cylindrocarpon liriodendri* J. D. MacDon. and E. E. Butler, *Cylindrocarpon destructans* (Zinsm.) Scholten, *Cylindrocarpon macrodidymum* Schroers, Halleen and Crous, and *Cylindrocarpon pauciseptatum* Schroers and Crous, affects grapevines throughout the main viticultural regions of the world (Halleen et al., 2006a, b).

Recently, a relatively high occurrence of vine decline has been observed in Iran. In 1998 and 1999, a grapevine disease with external and internal symptoms similar to esca was observed in Bojnourd (North Khorassan Province, north-eastern Iran). Association of *F. punctata* and *Pa. chlamydospora* was revealed with white decay and brown-red borders (Karimi et al., 2001). In May–June 2003, several vineyards were partly surveyed in

different areas of Iran, including Qom (Qom Province, north Iran), Shahrud (Semnan Province, north Iran) and Qazvin (Qazvine Province, north-western Iran). *Paeomoniella chlamydospora*, *Pm. aleophilum* and *Pm. parasiticum* W. Gams, Crous and M. J. Wingf. were found to be in association with diseased grapevines (Gräfenhan and Gams, 2004). Karimi-Shahri and Farashiani (2006) observed *Fomitiporia* sp., *Acremonium* sp., and *Phaeoacremonium* sp. in grapevines showing esca symptoms in the north of Khorassan Province. A survey conducted by Gräfenhan (2006) revealed that several fungi, *Pm. aleophilum*, *Pm. parasiticum*, *F. mediterranea*, *Pa. chlamydospora* and ‘*Phaeoacremonium* sp.’ were associated with vine decline symptoms. These ‘*Phaeoacremonium* sp.’ isolates were later identified as *Pm. iranianum* L. Mostert, Gra f., W. Gams and Crous (Mostert et al., 2006). In 2004, a field survey was carried out in different vineyards in Fars Province (south-western Iran), different fungi including *Pa. chlamydospora*, *Pm. aleophilum*, *Fusarium* sp., *Phialophora* sp., *Phoma* sp., *Phaeoacremonium* sp. and *Natrassia* sp. were recovered (Mohammadi and Banihashemi, 2007). This study is a step towards a greater understanding of grapevine decline disease in the regions still unexplored in this country. The aim of this work was to identify and characterize the causal agents of grapevine decline using both morphological and molecular methods with emphasis on the occurrence of *Phaeoacremonium* spp. and *Pa. chlamydospora* and as well as determination of their pathogenicity.

MATERIALS AND METHODS

Survey and Sample Collection

A survey of 41 own rooted grapevine vineyards (4 to 35 years old) in different production areas of Iran namely: Hamedan (middle-western Iran), Fars, Kohgiluyeh and Boirahmad (south-western Iran) and Isfahan (central Iran) provinces was conducted from 2004 to 2007 to identify the main

pathogenic grapevine wood fungi. At least 4-5 diseased grapevines from each own root ('Askari', 'Rishbaba' and 'Black' cultivars) showing yellowing and reduced growth plus different symptoms in the wood, including browning of wood, necrosis, brown and black streakings as well as white rot were sampled out. Infected crown, trunks and branches of diseased grapevines were cut into disks and surface disinfected by being immersed in 1.5% solution of NaOCl for 30 seconds and then rinsed in sterile distilled water (SDW). About 10 wood pieces of tissue were taken from the margin between necrotic and apparently healthy tissue and plated onto malt extract agar (MEA, 2% malt extract, Mashhad, Iran; 1.5% agar, Merck, Germany) supplemented with 1 g l^{-1} streptomycin sulphate (MEAS). Plates were incubated at 25°C in the dark for 2 weeks, with all colonies being transferred to potato dextrose agar (PDA; Merck, Germany). They were single-spored prior to morphological and molecular identification.

Fungal Identification

Morphological Identification

Isolated fungi were initially identified on the basis of morphological characteristics of the colonies and their reproductive structures. *Phaeoacremonium* species were identified based on their cultural characteristics and pigment production on PDA, MEA and oatmeal agar (OA; 30 g oatmeal; 15 g agar; Merck, Germany). Microscopic observations including conidiophore morphology, phialide type and shape and hyphal warts size from aerial mycelia of the colonies were made on MEA. Radial growth of isolates were recorded following 16 days at 25°C. (Mostert *et al.*, 2006). *Phaeomoniella chlamydospora* was identified through conidiophore morphology and colony characteristics on PDA and MEA (Crous and Gams, 2000). *Cylindrocarpon* isolates were transferred to PDA and presumptively identified as

Cylindrocarpon spp. through morphology and conidial characteristics (Booth, 1966). 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) through an attachment of a 1×1 cm piece of filter paper to the colony surface (Alaniz *et al.*, 2007). Species of Botryosphaeriaceae were identified through colony as well as conidial morphology (Phillips, 2006). To enhance sporulation, pure cultures were placed on 2% water agar (WA, 2% agar; Merck, Germany) containing autoclaved grapevine wood chips, incubated at 25°C under 12 hours photoperiod. Isolates were examined weekly for formation of pycnidia and conidia. Conidial morphology from pycnidia was recorded using a compound microscope. Fifty microscopic measurements of each type of the structures were made for all the studied isolates.

Molecular Identification

For DNA extraction, isolates were grown on PDA for 10–15 days at 25 °C in the dark. For each isolate approximately 50 mg of fungal mycelia were scraped from the surface of cultures and mechanically disrupted by being ground into 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, USA) following the instructions of the manufacturer. DNA samples were kept at –20°C until being for PCR amplification. The specific primers Pm1 and Pm2 for *Phaeoacremonium*, which yielded a fragment of 415 bp for the ITS1 and ITS2 regions of rDNA, were utilized for direct PCR amplification and detection of the genus *Phaeoacremonium* as described by Aroca and Raposo (2007). Identification of *Phaeoacremonium* species was achieved by digesting the PCR product amplified by Pm1 and Pm2 primers with three restricting enzymes namely: *Bss*KI, *Eco*O109I, and



*Hha*I. *Bss*KI was used for separation and identification of *Pm. aleophilum*, while *Eco*O109I and *Hha*I for detection and separation of other *Phaeoacremonium* species from each other (Aroca and Raposo, 2007). *Phaeomoniella chlamydospora* was detected through PCR making use of primers Pch1-Pch2 (Tegli et al., 2000). In addition, partial sequences of the β -tubulin gene were amplified utilizing primers T1 (O'Donnell and Cigelnik, 1997) and Bt2b (Glass and Donaldson, 1995) to confirm *Phaeoacremonium* spp. and *Pa. chlamydospora*. The PCR reaction contained 1× PCR buffer, 2.5 mM MgCl₂, 200 μM each dNTPs, 0.4 μM of each primer, 1 U of *Taq* polymerase (Dominion MBL, Córdoba, Spain), as well as 1 μl of template DNA. The PCR reaction mix was adjusted to a final volume of 25 μl with water (Chromasolv Plus, Sigma-Aldrich, Steinheim, Germany). PCR amplifications were performed on a Peltier Thermal Cycler-200 (MJ Research). DNA amplifications were carried out through the following program: (i) an initial denaturation step at 94°C for 5 minutes; (ii) 40 cycles, consisting of denaturation (30 seconds at 94°C), annealing (30 seconds at 52°C), and extension (50 seconds at 72°C); and (iii) a final extension step of 7 minutes at 72°C. Identification of Botryosphaeriaceae species was confirmed by the PCR protocol described by Slippers et al. (2004) using the pair of primers EF1-728F and EF1-986R (Carbone and Kohn, 1999). For *Cylindrocarpon* species identification, partial sequences of the BT gene, BT1, were amplified using primers BT1a and BT1b (Petit and Gubler, 2005). PCR products were analyzed through electrophoresis on 1.5% agarose gels (agarose D-1 Low EEO, Conda, Madrid, Spain) in TAE buffer and visualized by being stained with ethidium bromide. Positive as well as negative controls were included in each test. A 100 bp ladder was used as a molecular weight marker (Dominion MBL). PCR products were purified with the High Pure PCR Product Purification Kit (Roche Diagnostics,

Germany) and sequenced in both directions by the DNA Sequencing Service of the Universidad Politécnica de Valencia-CSIC.

Pathogenicity Tests

In March 2006, two isolates of *Pm. aleophilum* (Pal2-A and Pal2-B, GenBank accession nos. GQ903709 and GQ903710), *Pa. chlamydospora* (Pch-2 and Pch-3, GenBank accession nos. GQ903724 and GQ903725), *Pm. parasiticum* (Ppm-A and Ppm-B, GenBank accession nos. GQ903714 and GQ903715) and *Pm. inflatipes* (Pin-1 and Pin-2, GenBank accession nos. GQ903719 and GQ903720 respectively) were selected for pathogenicity tests under field conditions. A vineyard of 20-year-old vine plants cv. 'Askari' was selected at Shiraz University Experimental Station in Kooshkak. Thirty-two vines were randomly selected and surface-disinfected with 70% ethanol before being inoculated. For each isolate, 4 vines (3 branches in each vine) were used for pathogenicity tests. Inoculation was made by placing a 4 mm diameter mycelial plug into artificial wounds and protected by moist cotton while being wrapped with Parafilm®. Twelve branches were inoculated with 4 mm of noncolonized MEA agar plugs for negative controls. Inoculated branches were collected after 10 months and inspected for lesion development. Extent of vascular discoloration was recorded upwards as well as downwards from the inoculation point. Ten small pieces (about 0.5 cm) of necrotic tissue from the edge of each lesion were cut and placed on MEA in an attempt to recover the inoculated fungi and complete Koch's postulates. Fungi were identified as previously described. One-way analysis of variance (ANOVA) in SAS Ver. 9.1 (SAS Institute, Cary, North Carolina, USA) was performed in order to evaluate differences in the extent of vascular discoloration induced by fungal isolates. Student's *t*-test for Least Significant Difference (LSD) test was

carried out for a comparison of treatment means at $P < 0.01$.

RESULTS

Survey and Sample Collection

A total of 127 diseased plants belonging to three rootstock cultivars of 'Askari', 'Black' and 'Rishbaba' were sampled from 4 provinces in Iran (Table 1). Fungal trunk pathogens were isolated from only 98 plants (77.2%) as the positive samples. In most of the vineyards sampled, decline affected vines showed stunted growth, reduced foliage and small chlorotic leaves, slow die-back and plant death. Internal symptoms included wedge-shaped and central necrosis, brown to black streaking, black spots along with rare wood decay when vines were cut transversely, vs. dark brown to black streaking when trunks or shoots cut longitudinally (Figure 1). Esca symptoms such as "tiger-stripe" patterns on leaves were observed in 9 vineyards (21.95% of the surveyed vineyards) but typical esca symptoms including wood decay, white rot and black measles on berries were observed only in 3 vineyards (7.3% of the surveyed vineyards) in Bavanat (Fars province). During this study, only four plants in a vineyard (2.44% of the surveyed vineyards) in Bavanat, suffered from a sudden collapse (apoplexy) while some plants showed severe decline symptoms and eventually died. Most of the surveyed vineyards (78.05% of the surveyed ones) showed different Petri disease symptoms specially on 'Askari' cultivar while lower incidence of symptoms observed on other cultivars such as 'Black' and 'Rishbaba'.

Fungal Isolation and Identification

Two hundred and forty fungal isolates were obtained mainly from central wood and wedge-shaped necrosis and black spot areas of the trunks, shoots and branches of the

young (< 10 years old) as well as old (> 10 years old) vines. Several fungal trunk pathogens were isolated from diseased grapevines with different internal symptoms (Table 2, Figure1). Seventy four *Phaeoacremonium* isolates previously identified as based upon morphological and cultural characteristics were amplified using the primers pair Pm1 and Pm2. An amplicon of about 415 bp was obtained for all the *Phaeoacremonium* isolates. The three selected enzymes, *Bss*KI, *Eco*O109I, and *Hha*I, digested PCR products amplified using Pm1 and Pm2 primers. The first digestion which was carried out through *Bss*KI enzyme, separated *Pm. aleophilum* from other *Phaeoacremonium* species. The *Bss*KI-digested *Phaeoacremonium* amplicon produced a band of about 330 bp for *Pm. aleophilum* and a band of 250 bp for other species. Through this method, *Pm. aleophilum* isolates were identified from others. A second digestion was performed using *Eco*O109I to identify members of other species. The patterns consisted of two bands of 344 bp and 49 bp for some isolates that were previously (morphologically) identified *Pm. parasiticum* and two bands of 263 bp and 85 bp for other isolates. In order to further characterize these species, a third digestion with *Hha*I enzyme was done. *Pm. parasiticum* isolates showed a band of 295 bp while the other isolates produced a band of 241 bp. β -tubulin gene sequences of, *Pm. aleophilum* and *Pm. parasiticum* isolates from Iran showing 99 to 100% homology with *Pm. aleophilum* in GenBank (AF192390) and *Pm. parasiticum* (EU128081) deposited by Dupont *et al.* (2000) and by Damm *et al.* (2008), respectively. The sequences of Iranian *Pm. inflatipes* isolates were identical to that of *Pm. inflatipes* (AY579323) deposited by Mostert *et al.* (2006). The Pch1-Pch2 primer pair amplified a fragment of about 360 bp for 19 *Pa. chlamydospora* isolates, β -tubulin gene sequences of these isolates showing 99 to 100% homology with *Pa. chlamydospora*

**Table 1.** Geographical origin and number of fungal isolates recovered from diseased grapevine cultivars collected from Iran.

Species	Number of isolates	Cultivar	Year	Geographical origin		No ^a . (%)
				Province	Town	
<i>Phaeoacremonium aleophilum</i>	4	Askari	2007	Hamedan	Hamedan	45 (18.8%)
	1	Black	2006	Fars	Abadeh	
	3	Askari				
	5	Askari	2004		Kavar	
	2	Black	2005		Bavanat	
	6	Askari				
	2	Askari	2006		Eghlid	
	6	Askari	2005		Shiraz	
	2	Rishbaba				
	4	Askari	2007		Estahban	
	5	Askari	2005	Kohgiluyeh and	Yasuj	
	3	Askari	2007	Boirahmad	Sisakht	
	2	Askari	2006	Isfahan	Maghsoodbieg	
<i>Phaeoacremonium parasiticum</i>	2	Black	2005	Fars	Bavanat	19 (7.9%)
	6	Askari				
	7	Askari	2005		Kavar	
	4	Askari	2006		Abadeh	
<i>Phaeoacremonium inflatipes</i>	4	Askari	2007	Fars	Bavanat	4 (1.7%)
<i>Phaeomoniella chlamydospora</i>	1	Black	2007	Fars	Saadat Shahr	22 (9.2%)
	2	Rishbaba				
	4	Askari	2006		Abadeh	
	3	Askari	2004		Kavar	
	1	Black	2006		Bavanat	
	4	Askari				
	3	Askari	2007		Estahban	
	1	Askari	2007	Hamedan	Hamedan	
	3	Askari	2007	Isfahan	Shahrezah	
	10	Askari	2007	Fars	Bavanat	12 (5.0%)
<i>Neofusicuccum parvum</i>	2	Askari	2006		Kavar	
<i>Diplodia seriata</i>	15	Askari	2007	Fars	Bavanat	15 (6.3%)
<i>Cylindrocarpon liriodendri</i>	8	Askari	2005	Fars	Bavanat	8 (3.3%)
<i>Phaeoacremonium cinereum</i>	2	Askari	2007	Fars	Bavanat	6 (2.5%)
	2	Askari	2004		Kavar	
	2	Askari	2007		Abadeh	
	6	Askari	2007	Fars	Bavanat	8 (3.3%)
<i>Phoma</i> sp.	2	Black	2004		Saadat Shahr	13 (5.4%)
<i>Phialophora</i> sp.	2	Black	2007	Fars	Kavar	
	3	Askari				
	3	Rishbaba	2004		Shiraz	
	5	Askari	2007		Saadat Shahr	
	1	Rishbaba	2007	Fars	Bavanat	9 (3.8%)
<i>Acremonium</i> sp.	4	Askari				
<i>Fusarium</i> sp.	4	Black	2004	Isfahan	Shahreza	7 (2.9%)
	4	Askari	2007	Fars	Bavanat	
	3	Askari	2005	Kohgiluyeh and Boirahmad	Yasuj	
<i>Aspergillus</i> sp.	7	Askari	2007	Fars	Bavanat	10 (4.2%)
	2	Askari	2004	Isfahan	Shahreza	
	1	Askari	2007	Hamedan	Hamedan	
<i>Penicillium</i> sp.	1	Black	2007	Fars	Abadeh	12 (5.0 %)
	3	Askari				
	3	Askari	2005	Kohgiluyeh and Boirahmad	Yasuj	

(Continued...)

Table 1. (Continued)

Species	Number of isolates	Cultivar	Year	Geographical origin		No ^a . (%)
				Province	Town	
<i>Nattrassia</i> sp.	5	Askari	2007	Hamedan	Hamedan	4 (1.7%)
	1	Askari	2007	Fars	Bavanat	
	2	Black				
	1	Askari	2007	Hamedan	Hamedan	
<i>Paecilomyces</i> sp.	1	Askari	2007	Fars	Bavanat	5 (2.0%)
	2	Askari	2004		Kavar	
<i>Cladosporium</i> sp.	2	Askari	2007		Abadeh	15 (6.2%)
	2	Black	2007	Fars	Bavanat	
	3	Askari				
	2	Rishbaba	2004		Kavar	
Other phialidic fungi	1	Black	2005	Kohgiluyeh and Boirahmad	Yasuj	26 (10.8%)
	2	Askari				
	2	Black	2006	Isfahan	Shahreza	
	3	Askari				
	2	Askari	2007	Fars	Bavanat	
	3	Black				
	2	Rishbab				
	2	Black	2004		Kavar	
	4	Askari				
	8	Askari	2007	Kohgiluyeh and Boirahmad	Sisakht	
	3	Askari	2007	Hamedan	Hamedan	
	5	Askari	2007	Isfahan	Shahreza	

^a % from 240 total isolates.

isolates deposited in GenBank (AF253969, Groenewald *et al.*, 2001). Using PCR with the primers BT1a and BT1b, a fragment of about 470 bp was obtained for 8 *Cylindrocarpum* isolates. Based on phenotypical characteristics, mating experiments and molecular data, they were later identified as *C. liriodendri* (Mohammadi *et al.*, 2009). Using PCR with the primers EF1-728F and EF1-986R, a fragment of about 300 bp was obtained for 27 Botryosphaeriaceae isolates. Results of the sequencing and Blast search at GenBank showed that 12 isolates with hyaline conidia exhibited 99% homology with *Neofusicoccum parvum* (Pennycook and Samuels) Crous, Slippers and A. J. L. Phillips, isolates deposited at GenBank (AY343367, van Niekerk *et al.*, 2004) and 15 isolates with pigmented conidia showed 100% homology with those previously identified as *Diplodia seriata* De Not., at GenBank (EF173916, Cunningham *et al.*, 2007). Numerous isolates of *Aspergillus*

spp., *Penicillium* spp., *Cladosporium* spp., *Phialophora* sp., other phialidic fungi, and, less frequently *Fusarium* spp., *Phoma* sp. and *Acremonium* sp., were associated with diseased vines in different areas (Table 1). *Phaeoacremonium* species were isolated in 68.3% of positive samples (41.8% *Pm. aleophilum*, 17.3% *Pm. parasiticum*, 6.1% *Pm. cinereum* and 3.1% *Pm. inflatipes*) and 80.5% (33 vineyards) of the total surveyed vineyards (46.3% *Pm. aleophilum*, 22% *Pm. parasiticum*, 9.7% *Pm. cinereum* and 2.4% *Pm. inflatipes*). *Phaeomoniella chlamydospora* was isolated in 19.4% of positive samples and 29.3% (12 vineyards) of the total surveyed vineyards. The fungi most frequently isolated from symptomatic plants showing black spot and central necrosis were *Pm. aleophilum* (Hamedan, Fars, Kohgiluyeh & Boirahmad and Isfahan Provinces) and *Pa. chlamydospora* (Hamedan, Fars and Isfahan Provinces) with 18.8% and 9.2% of all isolations respectively (Table 1). Two species of *Pm.*

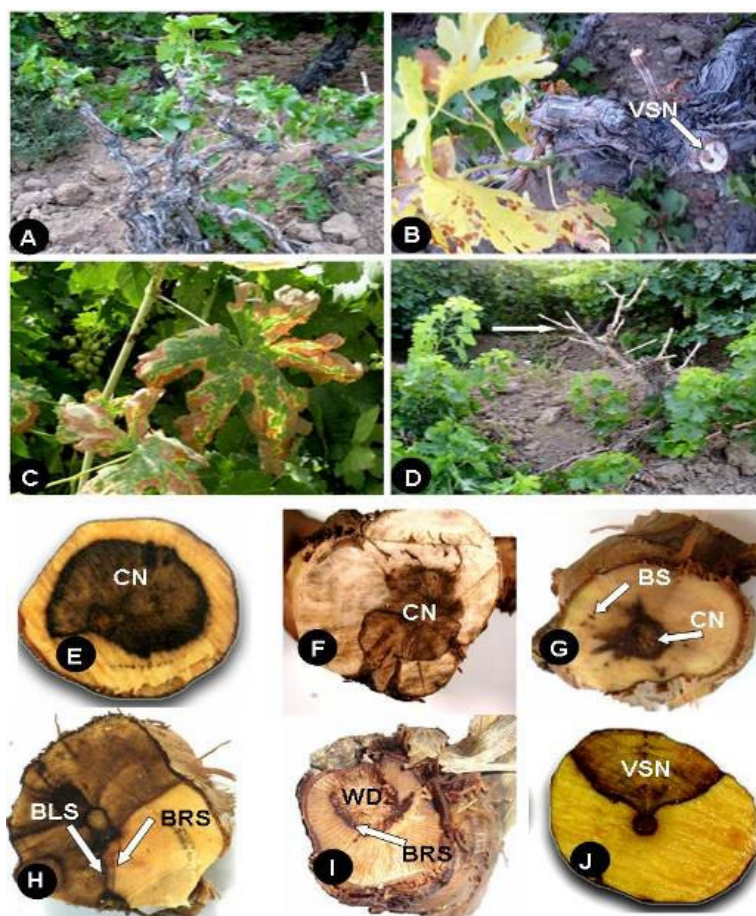


Figure 1. External and internal symptoms of grapevine trunk diseases observed in Iran: (A) Reduced growth and small leaves; (B) Co-occurrence of internal symptoms (V-shaped necrosis, shown by arrow) and external symptoms (leaf yellowing) on a 25-year-old vine in Kavar; (C) characteristic "tiger-stripe" patterns on leaves of a 30-year-old vine (Askari cv.) in Bavanat. (D) Grapevine decline disease symptoms; (E-J) Different internal symptoms, (E and F) Central necrosis (CN), (G) Co-occurrence of black spots (BS) and central necrosis; (H) Co-occurrence of wood discoloration and, black and brown streakings (BLS and BRS respectively, shown by arrows); (I) Cross section of a vine (Askari cv.) trunk showing wood decay (WD), characterized by a yellowish soft tissue, (J) Typical V- shape necrosis (VSN).

parasiticum and *Pm. inflatipes* (Fars province) were predominantly isolated from the central necrosis with 7.9% and 1.7% of the total isolations respectively. During this study, 6 isolates (2.5% of the total isolations) of a *Phaeoacremonium* sp. were isolated from cv 'Askari' collected from Kavar, Bavanat and Abadeh (Fars province) which were later identified as *Pm. cinereum*

D. Gramaje, H. Mohammadi, Z. Banihashemi, J. Armengol & L. Mostert (Gramaje *et al.*, 2009). Species of Botryosphaeriaceae were isolated in 31.9% of positive samples (5.1% *D. seriata* and 4.1% *N. parvum*) and 19.5% (8 vineyards) of vineyards. *Diplodia seriata* and *N. parvum* were predominantly isolated from the wedge-shaped necrosis, with 6.3% and

5.0% of the total isolations respectively. Eight isolates of *Cylindrocarpon* sp. were associated with the necrotic root lesions and trunk bases of 10-year-old grapevines in Bavanat. These isolates were obtained in 3.1% of positive samples and 4.9% (2 vineyards) of the total surveyed vineyards.

Pathogenicity Tests

Analyses of variance of the lesion length data on grapevine plants inoculated with *Phaeoacremonium* spp. and *Pa. chlamydospora* indicated a significant treatment effect ($F=962.71$ and $P<0.001$; ANOVA tables not shown). All fungal isolates used were pathogenic and produced internal vascular lesions on inoculated plants, which extended upward and downward from the point of inoculation, without any symptoms on leaves. *Phaeoacremonium chlamydospora* isolates were more virulent and produced significantly ($P<0.0001$) longer lesions (ranged from 175 to 240 mm) in all inoculated plants in comparison with those of *Pm. aleophilum* (ranging from 110 to 147 mm), *Pm. inflatipes* (ranging from 99 to 137 mm) and *Pm. parasiticum* (ranging from 95 to 121 mm) isolates. *Phaeoacremonium parasiticum* isolates produced smaller lesions than those caused by *Pa. chlamydospora* and other *Phaeoacremonium* isolates in all inoculated plants but still differed significantly from the control (ranging from 25 to 38 mm). No significant statistical difference was observed among two isolates in each species except in the two isolates of *Pm. inflatipes*. *Phaeoacremonium inflatipes* isolates exhibited significant differences ($F=12.18$, $P=0.0007$) in the extent of vascular discoloration with each other. Throughout the study one isolate of *Pm. inflatipes* (Pin-1) produced longer lesions and showed no significant differences with *Pm. aleophilum* isolates but other isolate (Pin-2) produced smaller lesions and showed no significant differences with *Pm. parasiticum* isolates.

Pathogen re-isolations from the lesion edges of all the inoculated plants yielded colonization characteristics of the species used for the inoculations, with none being recovered from the control plants. In this regard, *Pa. chlamydospora*, *Pm. aleophilum*, *Pm. inflatipes* and *Pm. parasiticum* were re-isolated from the inoculated plants with a frequency of 70 to 90%, 80 to 100%, 70 to 90% and 60 to 80% respectively.

DISCUSSION

Internal and external decline symptoms in most of the vineyards sampled were identical to those of Petri disease symptoms described in different countries (Mugnai *et al.*, 1999). However, esca symptoms including wood decay, white rot and black measles on berries were previously reported to commonly occur on vines in Khorassan (Karimi *et al.*, 2001) throughout in the present study, typical esca symptoms were observed only in 3 vineyards in Bavanat. Several fungi are known to cause trunk disease in grapevine. Two mitosporic fungi viz. *Pm. aleophilum* and *Pa. chlamydospora* are consistently isolated from diseased grapevines showing decline symptoms and internal wood discoloration (Scheck *et al.*, 1998; Mugnai *et al.*, 1999). The relative importance of the different *Phaeoacremonium* species in Petri disease and esca varied in different countries. Based on the obtained results, *Pm. aleophilum* and *Pa. chlamydospora* are the most frequently isolated species from vines showing decline symptoms in Iran. In this study *Pm. aleophilum* (48.8%) and *Pa. chlamydospora* (34.9%) were mostly isolated from black spots. In Spain, Luque *et al.* (2009) mostly isolated *Pa. chlamydospora* (73.1%) and *Pm. aleophilum* (12.4%) respectively from black spots and central necrosis sites. In Italy, *Pa. chlamydospora* was found in black spots and brownish zones (Mugnai *et al.*, 1996) while in France the fungus was associated with black line and brownish zones of the wood (Larignon and Dubos,



1997). The frequency of each fungal species isolated varied according to site. The results indicate that *Pm. aleophilum* was present in all provinces involved in this study. It seems that in Iran climatic conditions, this species is the main pathogen of grapevine causing Petri disease in the country as reported earlier (Mohammadi and Banihashemi, 2007) this being in agreement with the previous studies in Chile (Auger et al., 2005), Italy (Mugnai et al., 1996, 1999), France (Larignon and Dubos, 1997), South Africa (Groenewald et al., 2001), Spain (Armengol et al., 2001), Turkey (Ari, 2000), Yugoslavia (Crous et al., 1996) and the USA (Scheck et al., 1998). In some countries, *Pa. chlamydospora* is more often associated with typical Petri disease than species of *Phaeoacremonium* (Mugnai et al., 1999). Eight *Phaeoacremonium* species have been reported so far from grapevines in Iran namely: *Pm. aleophilum* (Gräfenhan and Gams, 2004, Gräfenhan, 2006, Mohammadi and Banihashemi, 2007), *Pm. parasiticum* (Gräfenhan and Gams, 2004; Mohammadi et al., 2008), *Pm. iraniana* L. Mostert, Gräf., W. Gams and Crous (Mostert et al., 2006), *Pm. viticola* J. Dupont (Gräfenhan et al., 2005; Gräfenhan, 2006), *Pm. cinereum* (Gramaje et al., 2009), *Pm. tuscanum* Essakhi, Mugnai, Surico and Crous (Mohammadi, 2011b), *Pm. inflatipes* W. Gams, Crous and M. J. Wingf., and *Pm. mortoniae* Crous and W. Gams (Mohammadi, 2011a). In the present study it has been shown that two Botryosphaeriaceae species namely *N. parvum* and *D. seriata*, are associated with grapevines showing decline symptoms in Iran. Botryosphaeriaceae species have been frequently isolated from grapevines showing decline or dieback symptoms in California (Úrbez-Torres et al., 2006a), Chile (Auger et al., 2004), Portugal (Phillips, 1998, 2002), Spain (Úrbez-Torres et al., 2006b), South Africa (Van Niekerk et al., 2004) and Australia (Castillo-Pando, 2001). Previous studies have demonstrated that external as well as internal symptoms of both eutypiose and black dead arm diseases are similar

(Castillo-Pando et al., 2001; Úrbez-Torres et al., 2006b, Luque et al., 2009). In France, Larignon and Dubos (1997) isolated *E. lata* more frequently than any botryosphaeriaceous fungus from wedge-shaped necrosis, whereas Armengol et al. (2001) and Úrbez-Torres et al. (2006a) proved a greater incidence of *D. seriata* than *E. lata* in Spain and California respectively. In the present study only *D. seriata* and *N. parvum* were predominantly isolated from the wedge-shaped necrosis sites. It seems that *E. lata* is less abundant in countries with dryer climates than in the cooler and more rainy regions; since *E. lata* dispersion is enhanced when mean annual rainfall exceeds 350 mm (Carter, 1991; Mugnai et al., 1999). In Iran *E. lata* was isolated only from grapevine in Arasbaran (in the northwest of Iran). In general, Iran suffers from an arid climate in which most of the relatively scant annual precipitation falls in October through April. The average annual rainfall in Iran is about 240 mm with maximum amounts in the Caspian Sea plains, Alborz and Zagross slopes with more than 1,800 and 480 mm, respectively. Pathogenicity of these Botryosphaeriaceae species was demonstrated in a study conducted on some 1-year-old grapevine cuttings cv. Cabernet Sauvignon with two isolates each of *D. seriata* (IRB2 and IRB7 isolates, Accession numbers, accession nos. GU121849 and GU121854 respectively) and *N. parvum* (IRN1 and IRN3, Accession numbers, accession nos. GU121863 and GU121865 respectively). Results indicated that *N. parvum* isolates were the most virulent and significantly differed from *D. seriata* isolates ($F=43.22$, $P<0.0001$) (Mohammadi et al., 2011).

Black foot disease caused by *Cylindrocarpon* spp. occurs as an important vine disease in all major viticulture regions worldwide, including Spain (Armengol et al., 2001), South Africa and New Zealand (Halleen et al., 2004). Previous studies have demonstrated that two species of *Cylindrocarpon*, *C. liriodendri* and *C. macrodidymum*, are isolated and reported as

the main causal agents of vine black foot disease (Halleen *et al.*, 2004; Halleen *et al.*, 2006a, b; Alaniz *et al.*, 2007). In Spain *C. macrodidymum* and *C. liriodendri* have been identified in association with young vines with *C. macrodidymum* being the predominant species (Alaniz *et al.*, 2007). In the present study it was only possible to isolate *C. liriodendri* with the necrotic root lesions and trunk bases of 10-year-old grapevines and this is the first report of this species with morphological and molecular details as the causal agent of black foot disease on grapevines in Iran. Pathogenicity test of these isolates was confirmed on 8-month-old dormant rooted cuttings of grapevine rootstock cv. 110 Richter (Mohammadi *et al.*, 2009) in agreement with previous results (Halleen *et al.*, 2004; Petit and Gubler, 2005; Alaniz *et al.*, 2007).

In the branch inoculations in the field, *Pm. aleophilum* and *Pa. chlamydospora* caused wood discoloration upward and downward from the point of inoculation after 10 months. *Pa. chlamydospora* produced significantly larger lesions than *Pm. aleophilum*. *Pa. chlamydospora* is obviously more aggressive than *Pm. aleophilum*. Halleen *et al.* (2007) also detected larger trunk and pruning wound lesions caused by *Pa. chlamydospora* after 14 months and considered it to be the most aggressive pathogen. Several previous studies also indicated higher symptom expressions by plants inoculated with *Pa. chlamydospora* than *Phaeoacremonium* spp. (Adalat *et al.*, 2000; Gramaje *et al.*, 2010). *Pa. chlamydospora* produced larger areas of vascular discoloration than *Phaeoacremonium* spp. under field (Mugnai *et al.*, 1999; Halleen *et al.*, 2007) and greenhouse (Halleen *et al.*, 2007; Aroca and Raposo, 2009) conditions.

Overall this research confirmed the importance of the fungal grapevine trunk pathogens associated with vine decline disease in Iran. The present study is the first report of *Pm. inflatipes*, *Pm. cinereum*, *C. liriodendri*, *D. seriata* and *N. parvum* causing grapevine decline in this country.

Recently two other new species of *Phaeoacremonium* viz. *Pm. mertoniae* and *Pm. tuscanum* (Mohammadi, 2011a, b) have been isolated from grapevine in Iran, thus, further extended studies may reveal even other new pathogens associated with grapevine decline disease in this country.

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قارچهای بیمارگر همراه با بیماریهای شاخه و تنه انگور در ایران

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چکیده

طی سال های ۸۶-۱۳۸۳ به منظور مطالعه قارچ های همراه با بیماری های شاخه و تنه انگور از باغ های مختلف در ایران بازدید به عمل آمد. از درختان انگور که دارای علائم زردی و کاهش رشد و علائم داخلی چوب مانند قهوه ای شدن و سیاه شدن بافت چوب، وجود رگه های سیاه و قهوه ای و پوسیدگی داخلی چوب بودند نمونه برداری شد. جداسازی عوامل قارچی از بافت های آلوده شاخه، تنه و طوقه درختان و با استفاده از محیط کشت عصاره مالت-آگار (MEA) حاوی یک گرم در لیتر از سولفات استرپتومیسین (MEAS) انجام شد. بر اساس خصوصیات ریخت شناسی و مولکولی گونه های *Phaeoacremonium (Pm.) aleophilum*، *Phaeoacremonium (Pm.) cinereum*، *Pm. inflatipes*، *Pm. parasiticum* و با درصد کمتری گونه های *Neofusicoccum parvum*، *Diplodia seriata*، *Cylindrocarpon liriodendri* از نمونه های بیمار جداسازی و شناسایی گردید. بر اساس نتایج حاصل از آزمون بیماری زایی در شرایط مزرعه ای، گونه های *Phaeoacremonium* و *Pa. chlamydospora* بر روی درختان مایه زنی شده بیماری زا بودند و پس از ۱۰ ماه باعث ایجاد تغییر رنگ بافت چوب شدند و هیچ گونه علائم برگری مشاهده نشد. در این میان *Pa. chlamydospora* نسبت به سایر جدایه ها بیماری زایی بیشتری را نشان داد. همه گونه های مایه زنی شده از حاشیه لکه های ایجاد شده مجددا جداسازی و شناسایی شدند. در این مطالعه برای اولین بار شناسایی مولکولی، مورفولوژیکی و بیماری زایی گونه های *Phaeoacremonium* و *Pa. chlamydospora* همراه با زوال انگور در ایران مورد بررسی قرار گرفت و همچنین این اولین گزارش از *Pm. inflatipes*، *N. parvum* و *D. seriata* همراه با زوال انگور در این کشور است.