First Report of Phaeoacremonium inflatipes and Phaeoacremonium mortoniae Associated with Grapevine Petri Disease in Iran

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ABSTRACT

Petri disease is responsible for grapevine decline and occurs wherever grapevines (Vitis vinifera) are cultivated. Phaeoacremonium species are among the principal hyphomycetes associated with Petri disease. During 2009, a field survey was conducted throughout different vineyards in the Fars province of Iran in order to determine the fungal pathogens associated with the vine decline observed in the region. Samples were taken from grapevines showing yellowing, interveinal chlorosis, leaf necrosis, reduced growth, wilting, wood necrosis and streaking, and xylem discoloration symptoms in cross section. Isolations were made from symptomatic wood tissues from cordons and trunks on malt extract agar supplemented with 1 mg ml\(^{-1}\) streptomycin sulphate (MEAS) and potato dextrose agar (PDA) media. Based on morphological and molecular characteristics two species of Phaeoacremonium, Phaeoacremonium mortoniae and Pm. inflatipes, were isolated and identified from grapevines showing yellowing, slow dieback, stunted growth, and reduced foliage in Bavanat (Fars province, south-western Iran). Pathogenicity tests were conducted on rooted grapevine cuttings (cv. Askari) under greenhouse conditions. Based on the results of pathogenicity tests, both tested Phaeoacremonium species were pathogenic and caused significant vascular discoloration in inoculated cuttings four months after inoculation. The fungi were reisolated from the margins of the lesion and healthy tissue, completing Koch’s postulates. Based on our knowledge, this is the first report of Pm. mortoniae and Pm. inflatipes causing grapevine Petri disease in Iran.

Keywords: Fars province, Vascular discoloration, Vine decline.

INTRODUCTION

Esca and Petri diseases are responsible for grapevine decline worldwide. Symptoms associated with Petri disease are characterized by stunted growth, shorter internodes, small leaves, interveinal chlorosis, smaller trunks and branches and a general decline of young vines resulting in plant death (Morton, 1995; Bertelli et al., 1998; Sidoti et al., 2000). Vascular symptoms can be seen by making cross and longitudinal sections in both cordons and trunk and include brown to black streaking of xylem tissues and black spots. The main pathogens associated with grapevine decline symptoms and Petri disease are Phaeoacremonium spp., most frequently Pm. aleophilum W. Gams, Crous, M.J. Wingf. and Mugnai [teleomorph: Togninia minima (Tul. and C. Tul.) Berl.], and Phaeomoniella chlamydospora (W. Gams, Crous, M.J. Wingf. and L. Mugnai) Crous and W. Gams (Larignon and Dubos, 1997). These species, in association with some basidiomycetes such as Fomitiporia mediterranea M. Fischer and to a lesser extent Stereum hirsutum (Willd.: Fr) Pers. are frequently reported as being the cause of esca in mature grapevines (Larignon and

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Dubos, 1997; Mugnai et al., 1999; Ari, 2000; Surico et al., 2006). Recently, five Phaeoacremonium spp. (Pm. armeniacum, Pm. globosum, Pm. occidentale A.B. Graham, P.R. Johnst. and B. Weir (Graham et al., 2009), Pm. cinereum D. Gramaje, H. Mohammadi, Z. Banihashemi, J. Armengol and L. Mostert and Pm. hispanicum D. Gramaje, J. Armengol & L. Mostert) were isolated and identified from grapevines (Gramaje et al., 2009a). To date, 25 species of Phaeoacremonium have been identified and reported from grapevines worldwide (Crous et al., 1996; Dupont et al., 2000; Mostert et al., 2005, 2006; Essakhi et al., 2008; Gramaje et al., 2009a, Graham et al., 2009).

In Iran, esca disease was first reported by Karimi et al. (2001). They observed foliar and trunk symptoms similar to esca disease in grapevines cultivated in Bojnourd (in the north of Khorassan province) during 1998-99. Fomitiporia mediterranea and Pa. chlamydospora were isolated from white wood decay and wood discoloration, respectively (Karimi et al., 2001). A similar study was conducted during 2005-06 in Northern-Khorassan province and several fungi including Fomitiporia sp., Phaeoacremonium sp., Acremonium sp. and Cephalosporium sp. were found in association with infected grapevines (Karimi-Shahri and Farashiyan, 2006). Thereafter, a general survey was conducted in different vineyards in various provinces of Iran including Fars, Isfahan, Kohgiluyeh and Boirahmad and Hamedan. Based on morphological and molecular characteristics, the following fungi were identified to be associated with symptomatic vines: Pm. aleophilum, Pm. parasiticum, Phaeoacremonium sp., Pa. chlamydospora, Diplodia seriata, Neofusicoccum parvum, Cylindrocarpon liriodendri, Phoma sp., P. iranianum, Pa. chlamydospora and P. cinereum (Gramaje et al., 2009a). Recently, isolates of Phaeoacremonium sp. were identified as Pm. aleophilum and Phaeoacremonium sp. (Mohammadi, 2008). Later, isolates of Phaeoacremonium sp. were identified as Pm. aleophilum, Pm. parasiticum, Phaeoacremonium sp., Pa. chlamydospora, Diplodia seriata, Neofusicoccum parvum, Cylindrocarpon liriodendri, Phoma sp., P. iranianum, Pa. chlamydospora and P. cinereum (Gramaje et al., 2009a). Recently, a relatively high occurrence of grapevine decline disease has been observed in different vineyards of Iran (Mohammadi and Banihashemi, 2007; Mohammadi et al., 2008; Banihashemi et al., 2009; Mohammadi et al., 2009). The aim of this study was to identify isolates of Phaeoacremonium collected from young vines in Fars province that appeared to be morphologically different from known Phaeoacremonium species in Iran.

MATERIALS AND METHODS

Field Survey and Sample Collection

During spring and summer of 2009, a field survey of 28 own rooted grapevine vineyards, between 4 and 35 years old, was conducted in the Fars province (south-western Iran) in order to determine the fungal pathogens associated with vine decline. Five to seven samples were taken from each vineyard from grapevines showing yellowing and necrotic spotting of the leaves, reduced growth of the canes and shoots, defoliation, and different symptoms in wood such as black spots, central brown necrosis, brown and black streaking of the wood and white rot.

Fungal Isolation and Identification

Different parts of grapevines including symptomatic crown, mid-trunk and branches were used for isolation. Cross and longitudinal sections of woody vine parts were examined in order to see the presence of wood discoloration symptoms. Isolation was made from different types of necrotic
tissues. Small pieces of approximately 4 mm in size of symptomatic tissue were surface disinfected by immersing in 1.5% solution of NaOCl for 30 seconds, rinsed by sterile distilled water (SDW) and plated on malt extract agar (MEA: 2% malt extract, Merck, Germany: 1.5% agar, Merck, Germany) supplemented with 1 mg ml\(^{-1}\) streptomycin sulphate (MEAS). Cultures were incubated at 25°C in the dark. Isolates were transferred to potato dextrose agar (PDA: Merck, Germany) or MEA plates, incubated at room temperature and examined weekly.

**Morphological and Cultural Studies**

Morphological and cultural characters of single spore *Phaeoacremonium* isolates were studied on four media including MEA, PDA, water agar (WA, 2% agar; Merck, Germany) and oatmeal agar (OA: 30 g oatmeal; 12.5 g agar; Merck, Germany) (Dupont et al., 2000). To induce sporulation, isolates were cultured onto MEA and PDA and placed at 25°C in the dark for about three weeks. Microscopic mounts were made from aerial mycelia 2–3 cm from the colony margin. Micro-morphological characters such as conidiophore structure and size, phialide types and size, extent of wart formation, and conidial shape and size were measured/recorded from water mounts. Thirty measurements of each type of structure were made using a light microscope. Water agar was used to examine the presence and size of hyphal warts. Radial growth of the isolates was measured on MEA, PDA and OA after 16 days at 25°C (Mostert et al., 2006).

**Molecular Identification**

**DNA Extraction and Polymerase Chain Reaction (PCR)**

Isolates were grown on PDA for 10 to 15 days at 25°C in the dark. Fungal mycelia and conidia from pure cultures were scrapped and mechanically disrupted by grinding to a fine powder under liquid nitrogen using a mortar and pestle. Total DNA was extracted using the DNeasy Kit (Qiagen, Germany) following manufacturer recommendations. DNA samples were kept at -20°C until they were used for PCR amplifications.

The specific primers Pm1 and Pm2 for *Phaeoacremonium*, which yielded a fragment of 415 bp for the ITS1 and ITS2 regions of rDNA, were used for direct PCR amplification and detection of the genus *Phaeoacremonium* as described by Aroca and Raposo (2007). In addition, partial sequences of the β-tubulin gene, were amplified using primers T1 and Bt2b for the identification of *Phaeoacremonium* species. The PCR was performed as described by Aroca and Raposo (2007).

**Pathogenicity Tests**

Two isolates of *Pm. inflatipes* (Pin-1 and Pin-2, GenBank GQ903719 and GQ903720 respectively), *Pm. mortoniae* (PMH1, GenBank Accession No. JF831449 and PMH2) and *Pa. chlamydospora* (Pch-2 and Pch-3, GenBank accession nos. GQ903724, and GQ903725 respectively), as positive control, were selected for pathogenicity tests under greenhouse conditions. Pathogenicity tests were conducted on rooted grapevine cuttings cv. Askari. Cuttings were cut into uniform lengths (about 35 cm) and wounded between the two upper internodes with a 4 mm cork borer. A 4 mm mycelium agar plug from a 16-day-old culture was placed in the wound. Wounds were wrapped with moist cotton and parafilm. Twelve cuttings per fungal isolate were used. Twelve cuttings were inoculated with 4 mm non-colonized MEA agar plugs for a negative control. Inoculated cuttings were planted immediately in individual pots, placed in a greenhouse at 25°C and watered as needed. Plants were arranged in a completely randomized design. After four months, cuttings were collected and inspected for lesion development. The extent of vascular discoloration was measured upward and downward 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. The 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. The LSD test was used for the comparison of treatment means at $P<0.01$.

RESULTS

Fungal Isolation and Identification

Morphological Identification

In the present study, nine *Phaeoacremonium* isolates were obtained from a 7-year-old vineyard (cv. Askari) in Bavanat. Of these, four isolates were isolated from black spot of three infected grapevines showing yellowing, slow dieback, reduced foliage and decline symptoms. Based on micromorphological and cultural characteristics the isolates were different from *Pm. aleophilum* and *Pm. parasiticum* which were reported earlier from Iran.

Colonies of these isolates were flat and white to gray on PDA and felty to powdery and gray on OA. Colonies on MEA were flat and brownish gray in the dark at 25°C. Conidia were hyaline, conidiophores short and usually branched. Phialides were terminal or lateral and mostly monophialidic. Based on these morphological characteristics, the isolates were identified as *Pm. inflatipes* (Mostert et al., 2006). Morphological characteristics are shown in Tables 1 and 2. Micro- and macro-morphological features are summarized in Figure 1.

Five isolates of a *Phaeoacremonium* sp. were also obtained from discolored vascular tissues of two diseased grapevines showing slow dieback, stunted growth, small chlorotic leaves and reduced foliage. Colonies of these isolates were flat and yellowish white on PDA and OA. Colonies on MEA were white-to-pale gray in the dark at 25°C for 16 days. Conidia were hyaline, conidiophores short, unbranched and often ending in a single terminal phialide. Phialides were terminal or lateral and mostly monophialidic. Based on the morphological characteristics, the isolates were identified as *Pm. mortoniae* (Groenewald et al., 2001; Mostert et al., 2006). Morphological characteristics are shown in Tables 3 and 4. Micro and macro-morphological features are summarized in Figure 2.

Molecular Identification

The DNA extracted from *Phaeoacremonium* isolates found in this study was amplified using the primers pair Pm1 and Pm2. An amplicon of about 415 bp was obtained for *Phaeoacremonium* isolates.
Table 1. Micro-morphological characters of *Pheocreamonium inflatipes* isolates after 16 days at 25°C in the dark.

<table>
<thead>
<tr>
<th>Isolate code</th>
<th>Conidial dimension</th>
<th>Phialide size (μm)</th>
<th>Conidiophore size (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Conidial size (μm)</td>
<td>L/W ratio</td>
<td>Type I</td>
</tr>
<tr>
<td>Pin1</td>
<td>3.0(3.9)5.0 × 1.0(1.0)2.0</td>
<td>3.9</td>
<td>3.0(7.8)15.5</td>
</tr>
<tr>
<td>Pin2</td>
<td>3.0(4.3)5.0 × 1.0(1.0)1.5</td>
<td>4.3</td>
<td>3.5(8.0)14.5</td>
</tr>
<tr>
<td>Pin3</td>
<td>3.0(3.8)5.0 × 1.0(1.2)2.0</td>
<td>3.2</td>
<td>4.0(7.6)14.0</td>
</tr>
<tr>
<td>Pin4</td>
<td>3.0(3.7)5.0 × 1.0(1.2)1.5</td>
<td>3.1</td>
<td>4.0(7.7)12.0</td>
</tr>
<tr>
<td>Mean</td>
<td>3.0(3.9)5.1 × 1.0(1.1)1.8</td>
<td>3.60</td>
<td>3.6(7.8)14.0</td>
</tr>
</tbody>
</table>

* Minimum, mean value and maximum size for length and width of 30 conidia; b Length/Width; c and d Minimum, mean (in brackets) and maximum size for 30 phialides and conidiophores measured.

Table 2. Colony growth rates of *Pheocreamonium inflatipes* isolates after 16 days, at 25°C in the dark.

<table>
<thead>
<tr>
<th>Isolate code</th>
<th>MEA</th>
<th>PDA</th>
<th>OA</th>
<th>MEA</th>
<th>PDA</th>
<th>OA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pin1</td>
<td>26.5(28–27.8)28</td>
<td>22.5(23–22.8)23</td>
<td>29.5(29.5–29.6)30</td>
<td>1.74</td>
<td>1.43</td>
<td>1.85</td>
</tr>
<tr>
<td>Pin2</td>
<td>25.5(27.5–27.2)28</td>
<td>21.2(23.5–22.9)24</td>
<td>28.5(28.5–29.1)30</td>
<td>1.69</td>
<td>1.43</td>
<td>1.82</td>
</tr>
<tr>
<td>Pin3</td>
<td>27(27.5–27.7)28</td>
<td>21.5(25.2–22.2)24</td>
<td>28.5(28.9–29.5)30</td>
<td>1.71</td>
<td>1.49</td>
<td>1.81</td>
</tr>
<tr>
<td>Pin4</td>
<td>28.5(29.5–29.4)30</td>
<td>23.5(24–24.1)25</td>
<td>30.1(31–31.1)32.5</td>
<td>1.84</td>
<td>1.51</td>
<td>1.94</td>
</tr>
<tr>
<td>Mean</td>
<td>26.9(28–27.9)28</td>
<td>22.1(23–23)24</td>
<td>29.1(29–29.7)30.5</td>
<td>1.75</td>
<td>1.44</td>
<td>1.86</td>
</tr>
</tbody>
</table>

* Minimum size, most frequent value and mean size (in brackets) and maximum size of radial growth.

Table 3. Micro-morphological characteristics of *Pheocreamonium mortoniiae* isolates after 16 days at 25°C in the dark.

<table>
<thead>
<tr>
<th>Isolate code</th>
<th>Conidial dimension</th>
<th>Phialide size (μm)</th>
<th>Conidiophore size (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Conidial size (μm)</td>
<td>L/W ratio</td>
<td>Type I</td>
</tr>
<tr>
<td>PMH1</td>
<td>2.0(5.0)7.0 × 1.5(1.5)2.0</td>
<td>3.3</td>
<td>3.0(7.8)15.5</td>
</tr>
<tr>
<td>PMH2</td>
<td>2.5(5.3)6.5 × 1.0(1.3)1.5</td>
<td>4.1</td>
<td>3.5(8.0)14.5</td>
</tr>
<tr>
<td>PMH3</td>
<td>2.0(4.5)6.5 × 1.0(1.4)1.5</td>
<td>3.2</td>
<td>4.0(7.6)14.0</td>
</tr>
<tr>
<td>PMH4</td>
<td>1.5(4.5)6.5 × 1.0(1.2)1.5</td>
<td>3.8</td>
<td>4.0(7.7)12.0</td>
</tr>
<tr>
<td>PMH5</td>
<td>2.0(5.0)6.0 × 1.0(1.6)2.0</td>
<td>3.1</td>
<td>4.0(7.7)12.0</td>
</tr>
<tr>
<td>Mean</td>
<td>2.0(4.9)6.5 × 1.0(1.1)1.7</td>
<td>3.5</td>
<td>3.6(7.8)14.0</td>
</tr>
</tbody>
</table>

* Minimum, mean value and maximum size for length and width of 30 conidia. b L/W = Length/width. c and d Minimum, mean (in brackets) and maximum size for 30 phialides and conidiophores measured.
Figure 2. Phaeoacremonium mortoniae. 16-day-old colonies on MEA (A) and PDA (B). Colonies reverse on MEA (C) and PDA (D). Type of phialides and conidiophore (E); type I (a); Type II (b); Type III (c); conidiophore (d), Conidia (F).

These isolates were also amplified by using T1 and Bt2b primers. A PCR fragment of about 750 bp was obtained from a partial sequence of their β-tubulin gene. After sequencing and Blast search in the GenBank, four isolates of Pm. inflatipes showed 100% similarity to the sequence of Pm. inflatipes isolates in GenBank (GenBank Accession No. AY579323, Mostert et al., 2005). The sequence of Pm. mortoniae isolate showed 100% similarity to corresponding sequence from Pm. mortoniae deposited in GenBank (GenBank Accession No. HM116767, Johnston et al., 2010, unpublished).

**Pathogenicity Tests**

Analyses of variance of the lesion length data on grapevine cuttings inoculated with Phaeoacremonium species indicated a significant treatment effect ($F = 385.11$ and $P < 0.001$; ANOVA tables not shown). All fungal isolates used were pathogenic and...
caused large wood discoloration on inoculated plants, which were significantly longer than the controls. Pa. chlamydospora isolates were more virulent and produced significantly ($P< 0.0001$) longer lesions (ranging from 58 to 71 mm) in all inoculated plants than those of *Pm. mortoniae* (ranging from 41 to 56 mm) and *Pm. inflatipes* (ranging from 24 to 37 mm) isolates, while the discoloration of control treatments was scanty (ranging from 15 to 21 mm). *Pm. inflatipes* isolates produced smaller lesions than those caused by *Pa. chlamydospora* and *Pm. mortoniae* isolates in all inoculated branches but still differed significantly from the control. The fungi were reisolated from the margins of the lesion and healthy tissue, completing Koch’s postulates, while no pathogens were found in the control plants.

**DISCUSSION**

Members of the genus *Phaeoacremonium* are known to be cosmopolitan, having a range of woody hosts and wide geographical distribution. They are reported from grapevine from different countries including Italy (Mugnai et al., 1999), France (Dupont et al., 2000), Greece (Rumbos and Rumbou, 2001), Argentina (Dupont et al., 2002), Australia (Mostert et al., 2005), Chile (Auger et al., 2005), Austria (Reisenzein et al., 2000), Spain (Armengol et al., 2001), USA (Schek et al., 1998), South Africa (Crous et al., 1996), Turkey (Ari, 2000) and Iran (Grafenhan and Gams 2004; Mohammadi et al., 2008). In recent years, grapevine trunk diseases have gained importance in Iran. In the present study, molecular and morphological studies identified two species of *Phaeoacremonium*, *Pm. inflatipes* and *Pm. mortoniae*, to be associated with grapevines showing Petri disease symptoms in Bavanat. *Phaeoacremonium* species are often found during general surveys of grapevine trunk pathogens in other grapevine-growing countries (Mostert et al., 2006; Essakihi et al., 2008). Petri disease is a major cause of grapevine decline in Iran (Mohammadi et al., 2008).

Micromorphological characters, such as cultural characters, size of hyphal warts, conidiophore morphology, and phialide type and shape are useful for the identification of *Phaeoacremonium* species (Mostert et al., 2005; 2006). However, distinguishing species based solely on morphological characters has proven to be difficult and it has resulted in some misidentifications. The ability to rapidly and accurately identify pathogens that cause Petri disease and esca is a critical first step for epidemiological studies and for a better understanding of the distribution and importance of individual species. Therefore, molecular tools have contributed to identify *Phaeoacremonium* species. In this study based on *Pm1* and *Pm2* primers and β-tubulin sequencing data amplified by T1 and Bt2b primers, two species of *Phaeoacremonium*, *Pm. inflatipes* and *Pm. mortoniae*, were identified as the causal agents of Petri disease. *Pm. inflatipes* was originally described based on morphological and cultural characteristics by Crous et al. (1996). This species has been reported from grapevine in California (Scheck et al., 1998; Eskalen and Gubler, 2001), Costa Rica (Mostert et al., 2006) and Spain (Gramaje et al., 2009b). *Pm. mortoniae* Crous and W. Gams, was also identified and described based on morphological characters, the internal transcribed spacer (ITS) regions 1 and 2, the 5.8S rDNA (Dupont et al., 2000) and the β-tubulin gene (Groenewald et al., 2001). This species has been reported from grapevines in Hungary and Croatia (Essakihi et al., 2008), Spain (Gramaje et al., 2007), Sweden and USA (Mostert et al., 2006), from kiwifruit in Italy (Prodi et al., 2008) and from *Prunus salicina* in South Africa (Damm et al., 2008).

In this work, both tested *Phaeoacremonium* species caused significant vascular discoloration on wood in inoculated grapevines 4 months after inoculation, although none of them was more virulent than *Pa. chlamydospora*,
which caused the largest vascular discoloration affected inoculated area. Several previous studies also indicated higher symptom expression 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. In different pathogenicity studies, Pa. chlamydospora, Pm. aleophilum and Pm. inflatipes have been shown to induce decline of young grapevines (Scheck et al., 1998). Pm. inflatipes, Pm. aleophilum and Pa.chlamydospora are reported as the causal agents of young vine decline in California. These three species were shown to be pathogenic to grape seedlings and 1-year-old rooted grapevine cuttings (Scheck et al., 1998). In seedlings cv. Malvar, all Phaeoacremonium species caused defoliation with the exception of Pm. inflatipes, which caused stem necrosis (Aroca and Raposo, 2009). To date, two new species of Phaeoacremonium as Pm. iranianum (Mostert et al., 2006) and Pm. cinereum (Gramaje et al., 2009a) have been described from Iranian vineyards. This study provides evidence for the presence of two other Phaeoacremonium spp., Pm. inflatipes and Pm. mortoniae, as the causal agents of vine decline in Iran, and thus; future field surveys in this country may reveal the presence of other fungal pathogens especially within the Phaeoacremonium genus in addition to the fungi reported here.

REFERENCES


Phaeoacremonium و Phaeoacremonium inflatipes
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چکیده

بیماری پترو یکی از بیماری‌های زوال انگور Phaeoacremonium یکی از اصلی ترین هیفومیسته‌های همراه با انگور در دنیا می‌باشد. در سال 1388 بر اساس تحقیقی که در آذربایجان و سیستان کردند و در مورد بیماری این مورد بررسی شد که بیماری در زردی سبب وصول می‌شود. بر روی انگور و در بلوط‌های جنگل، شاخه‌های جنگلی و در زیر سطحی می‌گردد.


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