J. Crop Prot. 2012, 1 (2): 153-159 _____

Short paper

First report of *Phaeoacremonium rubrigenum*, associated with declining persimmon trees in Iran

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Abstract: The genus *Phaeoacremonium* is associated with decline disease of woody plants and with human infections. Members of this genus have broad host range and wide geographical distribution. During 2010, ten isolates of *Phaeoacremonium* were recovered from vascular tissues of persimmon (*Diospyros kaki*) trees, showing decline symptoms in Shiraz city. Anamorphic characteristics such as, conidiophore morphology, phialide type and shape, size of hyphal warts, conidial size and shape were investigated. Based on morphological characteristics the presence of *Pm. rubrigenum* in Iran was documented. To confirm morphological identification, DNA was extracted from isolates using a genomic DNA purification Kit. Region of internal transcribed spacers 1, 2 and 5.8S genes of rDNA were amplified using ITS4 and ITS1 universal primer set. Fragments of 630 bp were recovered from PCR, purified, sequenced, edited and deposited in GenBank. *Pm. rubrigenum* isolates had an average of 99 % identity with all *P. rubrigenum* sequences compared. This species is a new report from Iran.

Keywords: *Phaeoacremonium rubrigenum*, persimmon trees, molecular identification

Introduction

Phaeoacremonium, a hyphomycetous genus, was introduced in 1996 with *Pm. parasiticum* as its type species (Crous *et al*., 1996). Members of the genus *Phaeoacremonium* are known to be cosmopolitan, having broad host range and wide geographical distribution. According to Mostert *et al*., (2006) of 22 *Phaeoacremonium* species that have been identified, nine of them, were isolated and reported from humans (Crous *et al*., 1996; Mostert *et al*., 2005) and 13 species were reported from various plants and humans (Mostert *et al*., 2006). *Phaeoacremonium* spp. have been reported from various woody plants that were associated with decline, wilting and dieback symptoms, including oak (*Quercus virginiana*) in Texas (Halliwell 1966), *Nectandra* sp. in Costa Rica (Hawksworth *et al*., 1976), *Prunus* spp. in South Africa (Damm *et al*., 2005), cherry in Greece (Rumbos 1986), kiwifruit vines in Greece, France and Italy (Di Marco *et al*., 2004), *Fraxinus pensylvanica* in USA (Hausner *et al*., 1992), apricot (Hawksworth *et al*., 1976), date palm (*Phoenix dactylifera*) in Iraq and other trees, although, originally some isolates were mis identified as another fungus. *Pm. scolyti* has been isolated from larvae of *Scolytus intericatus* (oak bark beetle) and adults of *Leperisinus fraxini* (*Fraxinus excelsior* bark beetle) in Czech Republic (Kubatova *et al*., 2004). These isolates had first been identified as *Pm. rubrigenum*, but later they were re-identified as *Pm. scolyti* by Mostert *et al*., (2005). To date, there is no available information about the occurrence of dieback of persimmon trees and associated *Phaeoacremonium* species in Iran.

Handling Editor: Dr. Vahe Minassian

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Received: 17 June 2012, Accepted: 8 July 2012

Materials and Methods

Isolation

Cross and longitudinal sections of woody persimmon branches were examined in order to check for woody discoloration symptoms. Isolation was made from different types of necrotic tissues. Small pieces, approximately 4 mm in size, of discolored or decayed tissues were surface disinfected by immersing in 1.5 % solution on NaOCl for 30 sec, rinsed in sterile distilled water and plated on potato dextrose agar (PDA) and malt extract agar (MEA) amended with chloramphenicole (25 µg/ml).

Morphological and cultural studies

Morphological and cultural characters of *Phaeoacremonium* isolates were studied on four media including MEA, PDA, WA and oatmeal agar (OA). The microscopic features were measured using water mounts of the specimens. Fifty measurements of each type of structure were made using BioloMICSMeasure software. Radial growth of the isolates was measured on MEA, PDA and OA after 16 days at 25 °C. All isolates were identified by morphological and molecular methods (Crous *et al*., 1996; Dupont *et al*., 2000; Alanize *et al*., 2007; Slippers *et al*., 2007).

DNA extraction and amplification

For DNA extraction, isolates were grown on PDA for 10 to 15 days at 25 °C in the dark. Total genomic DNA was extracted using a genomic DNA purification Kit (Fermentas, UK) according to the manufacturer's instruction. The ITS regions of nuclear rDNA were amplified with the universal ITS1 and ITS4 primers (White *et al*., 1990) on a CORBETT RESEARCH model CG1-96 thermocycler.

Sequencing of the amplified ITS regions

The amplification products of all specimens were purified with GeneJET PCR purification Kit (Fermentas, UK) to remove excess primers and nucleotides. Sequencing reactions were performed on purified PCR products in forward and revarse orientation using the primers used for amplification (ITS1 or ITS4). The sequence was determined with an ABI prism 377 DNA sequencer according to the manufacturers instruction. All DNA sequences of the ITS regions deposited at the National Center for Biotechnology Information GenBank (NCBI, http://www.ncbi.nlm.nih.gov/Entrez) (Bethesda, MD, USA).

Phylogenetic analysis

Sequences of the internal transcribed spacer regions including the 5.8S gene of rDNA were used to study phylogenetic relationships of the studied isolates. The internal transcribed spacer sequences of rDNA generated in this study were compared to those of other isolates obtained from GenBank (Fig. 3). Multiple alignments were performed with CLUSTALW (Thompson *et al*., 1994) using default settings and were manually optimized with BIOEDIT v.7.0.9 (Hall 1999). Phylogenetic analyses were performed by means of distance and maximum parsimony (MP) methods. Distances were calculated according to the Kimuras 2 parameter model (K2P). Tree topology was inferred by the Neighbor-Joining (NJ) method (Saitou and Nei 1987). The confidence of branching was assessed by computing 1000 bootstrap resamplings (Felsenstein, 1985). Maximum parsimony (MP) trees were inferred with the Close-neighbor-interchange (CNI) method with the aid of MEGA 4 software. The bootstrap method (Felsenstein, 1985) was performed with 1000 replications to evaluate the reliability of tree topologies. Other statistics, including tree length, consistency index, retention index and rescaled consistency index were calculated. *Pleurostomophora richardsiae* (GenBank accession no. AB364701) was selected as outgroup taxa based on their phylogenetic position in Essakhi *et al*., (2008).

Results

Fifty fungal isolates were recovered from persimmon trees showing decline and dieback symptoms. The most common fungi isolated from most diseased persimmons were *Pm. rubrigenum, Acrostalagmus luteoalbus* and *Lecanicillium lecanii* with the frequency of 25.2 7.3 and 6.3 percentage respectively. Numerous isolates of

Aspergillus spp., *Penicillium* spp. *Acremonium* sp. *Fusarium* spp. and *Rhizopus* sp. were always associated with diseased persimmons in Shiraz city. *Pm. rubrigenum* was isolated mainly from brown and black vascular tissues of branches.

Morphological identification

Ten isolates of *Phaeoacremonium* were obtained from discolored vascular system of persimmon trees. These isolates were characterized as follows: color of colony of isolates on malt extract agar (MEA), was medium pink and radial growth more than 9 mm after 7 d at 25 °C in the dark. Minimum temperature for growth 12 °C, optimum 27 °C, maximum 33 °C. Colonies on PDA flat, appressed and woolly to powdery. Colonies on OA flat and felty (Fig. 1). Phialides terminal or lateral, mostly monophialidic, smooth to verruculose, pale brown to subhyaline. Type I phialides subcylindrical, or elongated ampulliform, attenuated at the base, or constricted, 8–11 × 2-2.5 µm (Fig. 2 A); type II phialides mostly subulate, some navicular, tapering towards the apex, 15–18 × 2–3 µm (Fig.2 B and C). Type III phialides subulate, tapering towards the apex, 25-28 × 2-3 µm (Fig.2 D). Conidia hyaline, mostly ellipsoidal, some cylindrical, 3.5–4 × 1-2 µm (Fig. 2 E).

Molecular study

All *Phaeoacremonium* isolates previously identified based on morphological and culture characters, were amplified using the primers pair ITS1 and ITS4. A amplicon of about 600 bp was obtained for all of the *Phaeoacremonium* isolates. Through Blast search in GenBank all isolates were identified as *Pm. rubrigenum.* All DNA sequences of *Phaeoacremonium* isolates (accession numbers: JQ387572 and JQ387573) showed 100 % homology with valid sequences previously identified and deposited in GenBank. According to DNA sequence analyses and morphological characters, our isolates recovered from wood of *Diospyros kaki* showing dieback symptoms could be assigned to *Pm. rubrigenum* (Fig. 3).

Phylogenetic analysis

For the ITS data set, 71 sequences of *ca* 600 bp covering the ITS1 + 5.8S + ITS2 regions were used. The *Pleurostomophora richardsiae* was used as out group. Both distance-based and cladistic methods were applied for phylogenetic reconstruction of 71 specimens. The ITS phylogenetic trees inferred by both distance-based (Fig. 3) and cladistic methods (data not shown) showed the same topology, although there were differences in percent bootstrapping. In cladistic method, the tree length was 3958 with a CI of 0.507; R I = 0.82; RCI = 0.47 for all sites; iCI = 0.53 for parsimony informative sites and iRI = 0.82. With this, 47 trees were retained. Our isolates were clustered in a distinct monophyletic clade related to *Pm. rubrigenum* from other authors (Fig. 3). *Pm. rubrigenum* was a sister taxon of *Pm. scolyti* (98 % NJ and 99 % MP). Our *Pm. rubrigenum* isolates had an average of 99.5 % similarity with a range of 98.5-100 % similarity between themselves and only an average of 98 with a range of 93-100 % similarity between all *Pm. rubrigenum* sequences analyzed.

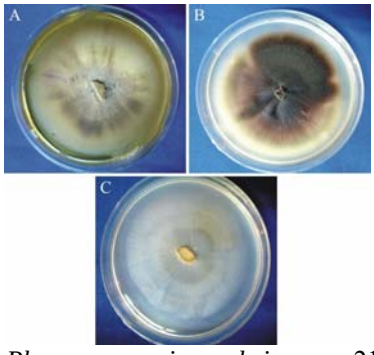

Figure 1 *Phaeoacremonium rubrigenum*. 21-day-old colonies on MEA (A), PDA (B) and OA (C).

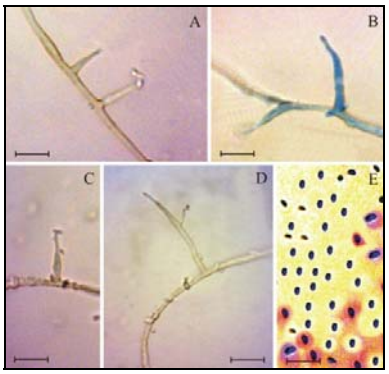

Figure 2 *Phaeoacremonium rubrigenum*. type I phialide (A), type II phialide (B and C), type III phialide (D), conidia (E). Bars = 25.6 µm (Figs A, B, C); 16.7 µm (Fig D); 25.6 µm (Fig. E).

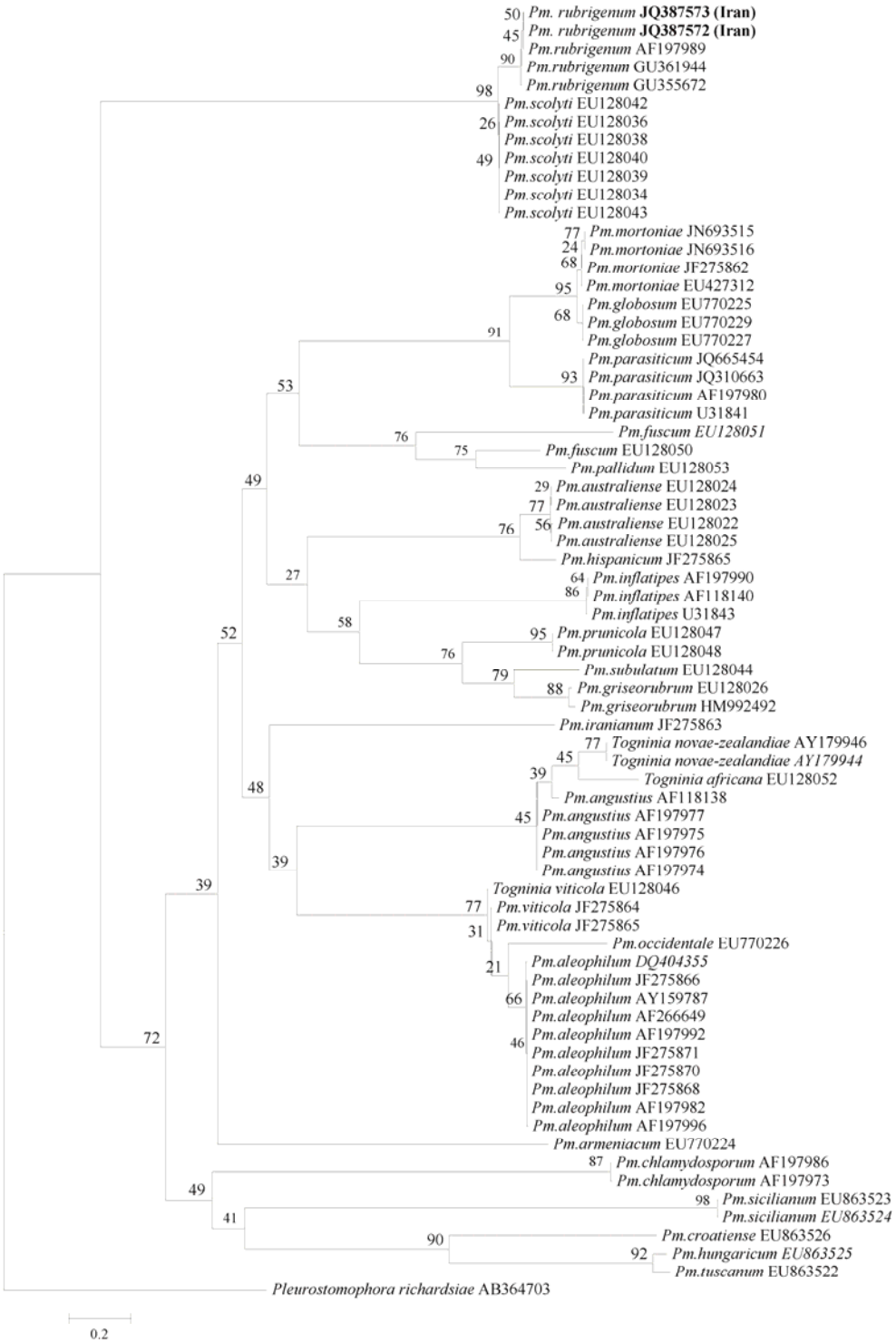

Figure 3 Neighbor joining phylogram generated in Mega from the alignment of 71 combined ITS1, 5.8S subunit, and ITS2 regions of the genomic ribosomal RNA sequences of *Phaeoacremonium* species using Kimura 2 parameter model with complete deletion gap handling and 1000-replication bootstrapping.

Discussion

Based on morphological characteristics 10 isolates of recovered fungi from diseased persimmons belonged to the genus *Phaeoacremonium*. But due to the overlappings in several characters among *Phaeoacremonium* species, some misidentifications have been made when using these characteristics and it seems that the use of molecular methods is needed in order to identify and separate different species correctly. Using PCR with the primers ITS1 and ITS4, a fragment of about 600 bp was obtained for *Pm. rubrigenum* isolates. Based on ITS gene sequences, these isolates showed 100 % homology with *Pm. rubrigenum* isolates deposited in GenBank. Subsequently, both phenotypical and molecular data confirmed the identification of the *Phaeoacremonium* isolates as *Pm. rubrigenum*. Analysis of sequence alignment shows that, in 71 *Phaeoacremonium* species compared in addition to *Pleurostomophora richardsiae* as an outgroup, there are 540 potentially phylogenetic informative sites which are mainly comprised of substitutions, deletions and insertions. Dupont *et al*., (2000) collected several *Phaeoacremonium* isolates and based on their morphology as well as DNA phylogeny of the transcribed spacers, 5.8 rRNA gene region and β-tubulin gene, designated these isolates as *Pm. viticola* (Dupont *et al*., 2000). Different molecular methods have been used for identification of *Phaeoacremonium* spp. such as; RFLP patterns, direct PCR based on specific primers and phylogenetic analysis data (Mostert *et al*., 2006). Tegli *et al*., (2000) used the RFLP patterns of ITS region for separation and identification of *Pm. inflatipes*, *Pm. aleophilum* and *Pm. rubrigenum* (Tegli *et al*., 2000). Later Dupont etal. (2002), used PCR-RFLP markers of ITS regions and analysis of the partial β-tubulin gene to distinguish five *Phaeoacremonium* species of *Pm. aleophilum*, *Pm. rubrigenum*, *Pm. inflatipes*, *Pm. viticola* and *Pm. parasiticum*. This is the first report of *Pm. rubrigenum* with morphological and molecular details in Iran. Previous studies show that *Pm. parasiticum*, *Pm. aleophilum*, and *Pm. inflatipes* are reported from Iran (Mohammadi and Banihashemi 2010). Pathogenicity of this species on persimmon trees in greenhouse is under investigation.

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اولین گزارش از *Phaeoacremonium rubrigenum* همراه با درختان خرمالوی در حال زوال از ایران

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چکیده: *Phaeoacremonium* یک جنس هیفومیستی می‌باشد که به‌عنوان عامل زوال میزبان‌های چوبی و آلوده‌کننده انسان توصیف شده است. ده جدایه از این جنس از بافت آوندی درختان خرمالوی در حال زوال از مناطق مختلف شیراز جداسازی شد. خصوصیات ریخت‌شناسی جدایه‌ها شامل ریخت-شناسی کنیدیوفورها، شکل و نوع فیالید، اندازه‌ی زگیل هیف‌ها و شکل و اندازه‌ی کنیدیوم‌ها مورد بررسی قرار گرفت. براساس خصوصیات ریخت‌شناسی حضور گونه‌ی قارچی *Phaeoacremonium rubrigenum* در ایران مشخص شد، ضمن اینکه با استفاده از آنالیز فیلوژنتیکی نواحی یگانه‌ای توالی‌های جداکننده‌ی نسخه‌برداری شده‌ی داخلی ۱، ۲ و ژن ۵/۸ اس دی ان ای ریبوزومی صحت تشخیص براساس خصوصیات ریخت‌شناسی تأیید شد. این اولین گزارش از حضور این گونه برای ایران می‌باشد.

واژگان کلیدی: *Phaeoacremonium rubrigenum*، درختان خرمالو، تشخیص مولکولی