

## ISOLATION, IDENTIFICATION AND PATHOGENICITY OF BOTRYOSPHAERIACEAE AND *PHAEOACREMONIUM* SPECIES ASSOCIATED WITH DECLINE OF *PRUNUS* SPECIES IN IRAN

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### SUMMARY

Between 2011 and 2013, a field survey was undertaken on stone fruit trees in four provinces of Iran to determine the fungal trunk pathogens associated with decline diseases. Wood samples were collected from branches of apricot, peach, cherry, sour cherry and greengage trees showing yellowing, dieback, defoliation, canker, gummosis, decline and internal wood discoloration. Five *Phaeoacremonium* species, including *Phaeoacremonium minimum*, *P. parasiticum*, *P. viticola*, *P. tuscanum* and *P. krajdenii* and six species of Botryosphaeriaceae, namely *Diplodia seriata*, *D. mutila*, *Neofusicoccum parvum*, *Botryosphaeria dothidea*, *Spenceriartinsia viticola* and *Lasiodiplodia theobromae*, were isolated and identified based on morphology, culture characteristics and DNA sequence analyses. Pathogenicity tests of candidate fungi were conducted by artificially inoculating detached shoots of all host species with a pathogen-colonized agar plug then covered with Parafilm. Thirty days post inoculation, the length of the necrotic wood lesions was measured. *Neofusicoccum parvum* was the most virulent among the six Botryosphaeriaceae species tested. Of *Phaeoacremonium* species, *P. parasiticum* produced the longest necrotic lesions on all inoculated hosts. The following are first reports from this study: (i) *P. tuscanum* on peach trees; (ii) *L. theobromae*, *B. dothidea*, and *P. krajdenii* on apricot trees; (iii) *B. dothidea*, *S. viticola*, *D. seriata*, *D. mutila*, *P. parasiticum*, *P. viticola* and *P. minimum* on cherry; (iv) *D. seriata*, *P. minimum* and *P. parasiticum* on sour cherry and (v) *S. viticola*, *L. theobromae* and *D. seriata* on greengage. *Phaeoacremonium krajdenii* has not been reported from Iran yet and the present study is the first report of this species in this country.

**Keywords:**  $\beta$ -tubulin, ITS, fungal trunk pathogens, stone fruit trees.

### INTRODUCTION

Various fungal genera are known to cause trunk diseases of woody plants including *Prunus* spp., which are susceptible to many fungal trunk pathogens worldwide (Damm *et al.*, 2008; Slippers *et al.*, 2007; Gramaje *et al.*, 2012). Species of Botryosphaeriaceae Ces. and De Not. (Inderbitzin *et al.*, 2010; Slippers *et al.*, 2007; Gramaje *et al.*, 2012) and Togniniaceae Réblová, L. Mostert, W. Gams et Crous (Damm *et al.*, 2008; Gramaje *et al.*, 2012) are well-recognized fungal trunk pathogens on stone fruit trees. Various members of Botryosphaeriaceae, *Botryosphaeria*, *Diplodia*, *Neofusicoccum*, *Lasiodiplodia*, *Aplosporella*, *Dothiorella* and *Neoscytalidium*, have also been reported on *Prunus* spp. (Agarwal *et al.*, 1992; Slippers *et al.*, 2007; Inderbitzin *et al.*, 2010; Gramaje *et al.*, 2012). To date, 16 *Phaeoacremonium* spp., i.e. *P. parasiticum*, *P. minimum*, *P. scolyti*, *P. iranianum*, *P. africanum*, *P. pallidum*, *P. griseo-olivaceum*, *P. venezuelense*, *P. mortioniae*, *P. fuscum*, *P. prunicolum*, *P. viticola*, *P. griseorubrum*, *P. australiense*, *P. amygdalinum* and *P. vibratile*, have been isolated and reported from *Prunus* spp. (Hawksworth *et al.*, 1976; Rumbos, 1986; Hausner *et al.*, 1992; Réblová and Mostert, 2007; Damm *et al.*, 2008, Gramaje *et al.*, 2012; Olmo *et al.*, 2014).

As to Iran, several fungal groups have been reported in association with trunk diseases and decline of stone fruit trees, including *Rosellinia necatrix* (Hartig) Berl. (Scharif and Ershad, 1966), *Armillaria mellea* (Vahl: Fr.) Kummer (Mohammadi-pour, 2000), *Cytospora leucosperma* (Pers.) Fr. (Fotouhifar *et al.*, 2008), *Fomes fomentarius* (L.) Fr. (Saber, 1974), *Phellinus igniarius* (L.) Quel (Saber, 1974), *Nectria ochroleuca* (Schw.) Berk. (Mohammadi-pour, 2004), *Verticillium dahlia* Klebahn (Bakhtiari *et al.*, 2006) and *Calosphaeria pulchella* (Pers.) J. Schröt. (Arzanlou and Dokhanchi, 2013). However, little work has been done so far in Iran to investigate the occurrence of Botryosphaeriaceae (Payghami and Ahary, 2001; Alizadeh *et al.*, 2000) and *Phaeoacremonium* spp. (Arzanlou *et al.*, 2014; Mousavi *et al.*, 2014) on stone fruit trees while most studies have focused mainly on grapevine trunk pathogens (Mohammadi *et al.*, 2013a, 2013b; Mohammadi and Hashemi, 2015).

In spring of 2011, a progressive dieback of branches associated with gummosis was observed on some aged trees (25-30-year-old) of peach and apricot in one orchard in Kerman (Kerman province, South-eastern Iran). In

branches with dieback and gummosis symptoms, wedge-shaped and irregularly-shaped necroses were generally observed when symptomatic parts were cross-sectioned. Upon further investigations, other disease symptoms such as yellowing, canker, defoliation and various internal wood lesions were also observed in various stone fruit trees in Kerman, Isfahan, Yazd and Fars provinces. Therefore, the aim of the present study was to determine the diversity of *Phaeoacremonium* and Botryosphaeriaceae species on *Prunus* trees showing symptoms of decline in these regions of Iran.

## MATERIALS AND METHODS

**Field survey, disease symptoms and sampling.** In May 2011, wood samples from branches of peach (*Prunus persica*) and apricot (*Prunus armeniaca*) trees showing defoliation, gummosis and dieback were collected in one orchard in Kerman. Symptomatic trees were located next to a vineyard (40-year-old), where some Botryosphaeriaceae and *Phaeoacremonium* species had previously been isolated from grapevines showing yellowing and dieback symptoms (Arabnezhad and Mohammadi, 2012). Additional samples of woody branches of other *Prunus* spp. including cherry (*Prunus avium*), sour cherry (*Prunus cerasus*) and greengage (*Prunus domestica* subsp. *italica* var. *claudiana*) that were dead or showing yellowing, dieback, defoliation, canker and gummosis were collected from selected orchards in Isfahan (Central Iran), Yazd (Central Iran) and Fars (South-western Iran) provinces of Iran (Table 1). In this study, 46 orchards of stone fruit trees were surveyed and woody samples were collected from each symptomatic tree (1-2 branches per tree) and analyzed for internal wood symptoms. In total 124 woody samples were collected from 87 trees including 25 *P. persica*, 31 *P. armeniaca*, 18 *P. avium*, 8 *Prunus domestica* subsp. *italica* var. *claudiana* and 5 *Prunus cerasus* trees in the four provinces. Cross and longitudinal sections of affected branches of each plant were examined to record the type of wood necrosis.

**Fungal isolation and identification.** Isolations were made from discolored tissues of infected branches. For each lesion detected, 10-12 pieces of wood (5 × 5 mm) were cut from the margin of infected and healthy tissue. The pieces of wood were surface-sterilised for 1.5 min in a 1.5% sodium hypochlorite solution, washed twice with sterile distilled water and blotted dry on sterile filter paper. Surface-sterilised tissue were plated on malt extract agar (MEA, Merck, Germany) supplemented with 0.5 g/l of streptomycin sulphate (MEAS) (Sigma-Aldrich, USA). Plates were incubated at 25°C. All fungal isolates were sub-cultured onto fresh MEA and incubated at 25 ± 2°C. Single conidial cultures of *Phaeoacremonium* species were obtained by spreading conidia on the surface of MEA and incubating at 25°C. Single germinating spores were

transferred to fresh MEA plates. Pure cultures of Botryosphaeriaceae isolates were obtained by excising and transferring a hyphal tip to fresh MEA plates.

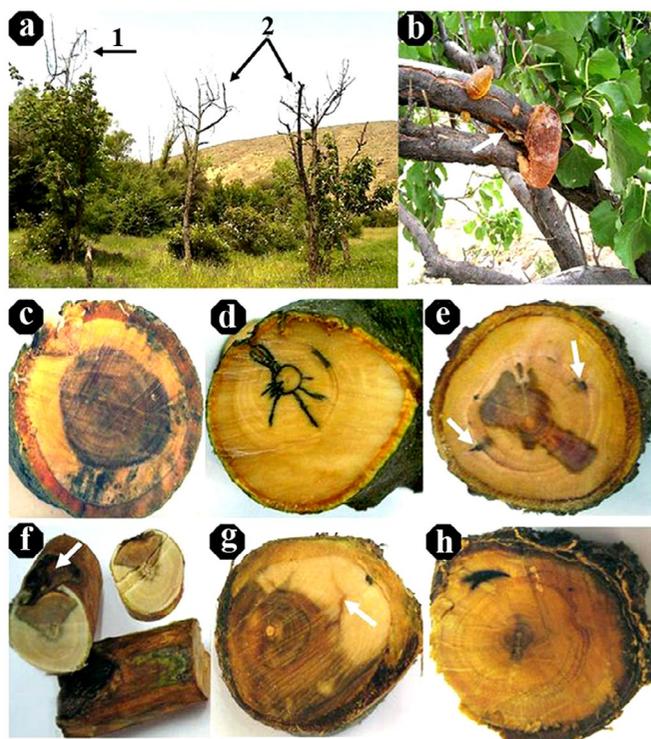
The morphological and culture characteristics were initially used to distinguish all fungal isolates obtained from symptomatic tissues. Species of *Phaeoacremonium* were identified according to their morphological characteristics and colony colour. Morphological characteristics used to distinguish species of this genus included type and shape of phialides, size and shape of conidia and size of hyphal warts. Colony characters and pigment production of *Phaeoacremonium* isolates on MEA, PDA and oatmeal agar (OA; Merck, Germany) at 25°C in the dark were recorded after 8 and 16 days as described in Mostert *et al.* (2006). Regarding Botryosphaeriaceae species, a detailed morphological study was carried out based on colony characters, conidial colour, shape and septation (Phillips, 2002, 2006; Slippers *et al.*, 2004). To induce sporulation, a 4 mm diameter plug of mycelium from each isolate was placed on 2% water agar medium (WA: 20 g agar/l) containing autoclaved pine needles (Van Niekerk *et al.*, 2004). The plates were incubated at 25°C in constant light to induce the formation of fruiting bodies and sporulation (Slippers *et al.*, 2004). Pycnidia and conidia produced on pine needles were mounted on microscope slides and examined under a light microscope. Fungal structures were mounted in lactic acid and measurements of 50 conidia and other fungal structures were measured using an Olympus BH2 microscope at ×1,000 magnification.

**DNA extraction, PCR amplification and DNA sequencing.** Total genomic DNA of selected isolates was extracted from pure culture mycelia cultivated on MEA using the Peq Gold Fungal DNA Mini Kit (Roche Diagnostics, Germany) following the manufacturer's protocol. The internal transcribed spacer (ITS) and a part of the translation elongation factor 1-alpha gene (EF1- $\alpha$ ) were amplified and sequenced with the primer pairs ITS1 and ITS4 (White *et al.*, 1990) and EF1-728F and EF1-986R (Carbone and Kohn, 1999), respectively. For Botryosphaeriaceae isolates, the protocols followed were as described by Úrbez-Torres *et al.* (2008). Morphological identification of *Phaeoacremonium* spp. was also confirmed by analysis of partial  $\beta$ -tubulin gene (BT) sequences amplified using primers T1 (O'Donnell and Cigelnik, 1997) and Bt2b (Glass and Donaldson, 1995), according to the conditions used by Mostert *et al.* (2005). PCR amplifications were performed on a Techne TC-312 Thermal Cycler (Techne, UK). Amplification products were analysed by electrophoresis through 1.0% agarose in TAE buffer. PCR products were purified with the High Pure PCR Product Purification Kit (Bioneer, Germany) and sequenced by Bioneer Corporation (Daejeon, South Korea). Edited sequences were run through BLAST /<http://blast.ncbi.nlm.nih.gov/Blast.cgi> to determine basic identity.

**Pathogenicity tests.** Selected isolates representing Botryosphaeriaceae and *Phaeoacremonium* spp. obtained from stone fruit trees were tested for pathogenicity on detached woody shoots of peach, apricot, sour cherry, greengage and cherry. Fresh vegetative shoots were collected from the trees (12- to 15-year-old) and cut into 30-35 cm pieces (5-7 mm in diameter). The outer bark at the inoculation area was cleaned and surface-sterilised with 70% ethanol. A mycelial plug (4 mm in diameter and 3 mm thick) taken from the margin of a 10-day-old fungal colony on PDA was placed into a 1 cm hole drilled radially with an ethanol-disinfected borer into a branch (at the second internode). The inoculated areas were protected by moist cotton and wrapped with Parafilm (Pechiney Plastic Packaging, USA) to prevent desiccation and contamination. Six shoots per each host were inoculated with non-colonised, sterile agar plugs as negative controls. The base of inoculated shoots were inserted into Erlenmeyer's flasks covered with parafilm, with 500 ml of sterilised tap water, then kept under glasshouse conditions at  $25 \pm 2^\circ\text{C}$ . Inoculated shoots were arranged in a completely randomised design (CRD). After 30 days, the bark surrounding each wound site was stripped off and the length of wood discoloration extending from the point of inoculation was measured. Re-isolation of fungal isolates was made from the margins of necrotic lesions onto MEA amended with streptomycin sulphate to fulfill Koch's postulates. The significance of differences in mean lesion lengths were determined by one-way analysis of variance (Proc ANOVA) using SAS v. 9.1 (SAS Institute, Cary, USA) and the LSD test was used for comparison of treatment means at  $P < 0.01$ .

## RESULTS

**Disease symptoms.** Sampled trees showed a variety of external symptoms including yellowing, branch dieback, canker and defoliation. In addition, some trees displayed severe decline and some died. Cankered areas often exuded a pale brown sap (gummosis), which gradually (within 6-7 days) dried to a blackish gluey mass on the bark (Fig. 1a). The occurrence of each external symptom varied from host to host and in a single orchard from tree to tree, depending on locations and the orchards surveyed. According to Fig. 1, six internal wood lesions were identified in stone fruit trees. These include central wood necrosis (Fig. 1c), arch-shaped necrosis, brown to black wood streaking (Fig. 1d), irregular-shaped necrosis, black spots (Fig. 1e) and wedge-shaped necrosis (Fig. 1f). Among all samples collected, central wood necrosis was the most prevalent wood discoloration observed (23.4% of the total samples collected) followed by wedge-shaped necrosis (16.9%), irregular-shaped necrosis (15.3%), black spots (11.3%), brown to black wood streaking (10.5%) and arch-shaped necrosis (2.4%). The remaining samples



**Fig. 1.** External and internal disease symptoms associated with trunk diseases of cherry, peach, greengage, apricot and sour cherry trees in Iran. a: dieback (1) and decline (2) symptoms on cherry in Kerman province; b: typical gummosis symptoms on apricot in Kerman province; c: central necrosis on peach in Yazd province; d: co-occurrence of arch-shaped necrosis and black wood streaking on sour cherry in Fars province; e: co-occurrence of irregular-shaped necrosis with black spots (shown by arrows) on greengage in Fars province; f: canker (indicated by arrow) and typical wedge-shaped necrosis on peach in Isfahan province; g: co-occurrence of irregular-shaped necrosis and brown wood streaking (shown by arrow) on cherry in Kerman province; h: arch-shaped necrosis on apricot in Fars province.

(20.2%) showed at least two different types of internal wood lesions on the same sample. In general, an external disease symptom was associated with several internal wood lesions and there was no clear relationship between external and internal disease symptoms. For example, in some apricot trees dieback was associated with all kinds of internal wood lesions in Kerman and Fars provinces. In contrast, in some cherry trees the same symptom was associated with central necrosis. Foliar and wood symptoms were quite common in some stone fruit tree orchards (Kerman and Fars provinces) but rare and, in some cases, absent in other surveyed orchards (Isfahan and Yazd provinces). The majority of diseased trees were found in old orchards (more than 12 years). Disease incidence in surveyed orchards ranged between 2-35% in all surveyed areas. The highest average of disease incidence was obtained from Kerman (20.5%) followed by Fars (16.3%), Yazd (12.8%) and Isfahan (11.3%).

**Table 1.** Details of origin, host, external and internal disease symptoms and fungal species obtained from *Prunus* spp. in Iran between 2011 and 2013.

Species	Stone fruit trees					Internal lesion types*						External symptoms**	Location (province)
	Peach	Apricot	Cherry	Sour cherry	Green-gage	AN	BL	BR	CN	IN	WN		
<i>Diplodia seriata</i>	9	2	5	2	1	2	2	3	2	1	9	Y, DI, GU, CA	Fars, Kerman
<i>Neofusicoccum parvum</i>	10	5	2	0	0	1	3	2	4	2	5	DI, GU, DE	Fars, Kerman, Isfahan, Yazd
<i>Botryosphaeria dothidea</i>	0	6	0	1	0	0	0	0	3	0	4	GU, DI, CA	Fars, Kerman
<i>Diplodia mutila</i>	3	4	0	2	0	0	3	0	0	4	2	DE, DI, CA	Fars, Kerman
<i>Lasiodiplodia theobromae</i>	0	3	0	0	2	0	0	0	3	0	2	DI, GU	Fars, Isfahan, Yazd
<i>Spencermartinsia viticola</i>	2	0	1	0	1	0	0	2	0	0	2	CA, GU, DI	Kerman, Yazd
<i>Phaeoacremonium viticola</i>	0	0	2	0	0	0	0	2	0	0	0	-	Fars
<i>Phaeoacremonium minimum</i>	10	9	7	3	0	1	5	2	12	4	5	DI, Y, DE	Fars, Kerman
<i>Phaeoacremonium parasiticum</i>	8	4	5	2	0	0	1	2	8	4	4	DI, CA, Y	Fars, Kerman
<i>Phaeoacremonium tuscanum</i>	3	0	0	0	0	0	0	0	1	2	0	DF	Kerman
<i>Phialopora</i> sp.	5	4	1	1	0	0	1	0	4	1	5	DI, DF, CA	Fars, Kerman, Isfahan, Yazd
<i>Phoma</i> spp.	0	8	0	0	1	0	1	0	0	2	6	CA, DI, GU	Fars, Kerman
<i>Phomopsis</i> sp.	2	1	0	0	0	0	0	0	0	0	3	CA, DI, Y	Kerman
<i>Paecilomyces</i> spp.	3	5	2	2	0	0	2	3	2	1	4	DI, CA, DF, DE	Fars, Kerman, Isfahan, Yazd
<i>Nattrassia mangiferae</i>	1	7	2	1	2	0	0	0	3	2	8	DI, DE, DF	Fars, Kerman, Yazd
<i>Phaeoacremonium krajdienii</i>	0	1	0	0	0	0	0	1	0	0	0	DI, GU	Fars
<b>Total</b>	<b>56</b>	<b>59</b>	<b>27</b>	<b>14</b>	<b>7</b>	<b>4</b>	<b>18</b>	<b>17</b>	<b>42</b>	<b>23</b>	<b>59</b>	-	-

\* Internal shoot symptoms: AN= Arch-shaped necrosis, BL= Black spots, BR= Brown to black streaking, CN=Central necrosis, IN= Irregular-shaped necrosis, WN= Wedge-shaped necrosis.

\*\* External disease symptoms: Y= Yellowing, DI= Dieback, GU= Gummosis, CA= Canker, DE= Decline, DF= Defoliation.

**Fungal isolation and identification.** A total of 163 fungal isolates were obtained from 93 necrotic wood samples (75% of the total samples). According to Table 1, most fungal isolates (36.2% of total isolates) were obtained from apricot trees, followed by peach (34.3%), cherry (16.6), sour cherry (8.6%) and greengage (4.3%). Totally, the most frequent fungal isolates (59/163 isolates) were isolated from wedge-shaped necrosis, amounting to 36.2% of the total isolates followed by central necrosis (25.8%), irregular-shaped necrosis (14.1%), black spots (11.0%), brown to black streaking (10.4%) and arch-shaped necrosis (2.5%).

Five species of *Phaeoacremonium* (54 isolates, representing 33.1% of total isolates) were obtained from stone fruit trees (17.8, 11.7, 1.8, 1.2 and 0.6% for *P. minimum*, *P. parasiticum*, *P. tuscanum*, *P. viticola*, and *P. krajdienii*, respectively). *P. minimum* and *P. parasiticum* (with the exception of greengage) were isolated from all investigated *Prunus* spp., while *P. viticola*, *P. tuscanum* and *P. krajdienii* were only isolated from cherry, peach and apricot, respectively. The highest incidence of *Phaeoacremonium* isolation was from central necrosis (21/54 isolates, 38.9%), followed by irregular-shaped necrosis (18.5%), wedge-shaped necrosis (16.7%), brown to black streaking (12.9%), black spots (11.1%) and arch-shaped necrosis (1.9%). Of the different *Phaeoacremonium* species, only *P. minimum* was isolated from all types of wood lesions while *P. viticola* and *P. krajdienii* were only isolated from brown to black wood streaking (Table 1).

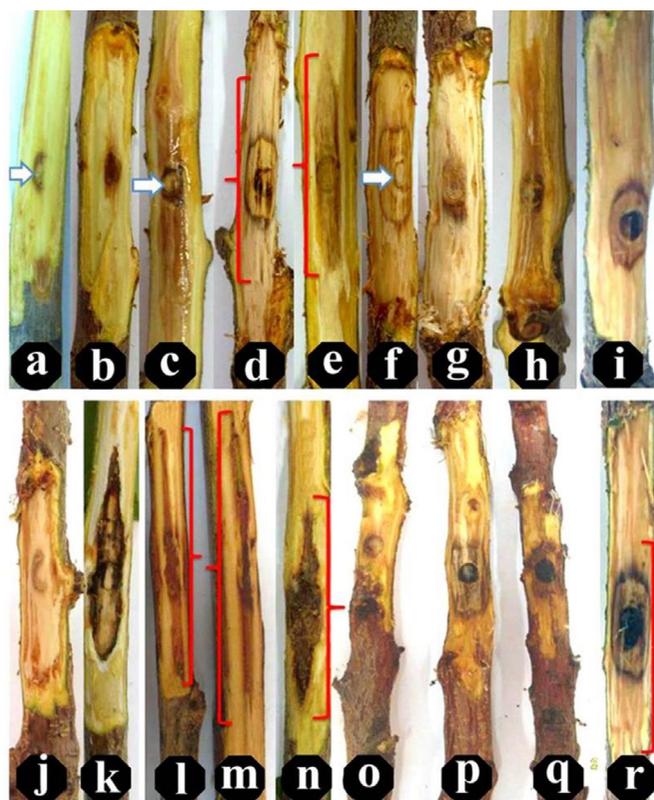
Sixty-one isolates of Botryosphaeriaceae (37.4% of total isolates) were isolated from diseased trees. Of these, *D. seriata* was the most prevalent species (11.7%), followed by

*Neofusicoccum parvum* (10.4%), *D. mutila* (5.5%), *Botryosphaeria dothidea* (4.3%), *Lasiodiplodia theobromae* (3.1%) and *Spencermartinsia viticola* (2.4%). Only *D. seriata* was isolated from all host species. *Neofusicoccum parvum* was obtained from peach, apricot and cherry, while *B. dothidea* was isolated only from apricot and sour cherry trees. *Diplodia mutila* was associated with peach, apricot and sour cherry, *L. theobromae* was obtained from apricot and greengage and *S. viticola* was detected on peach, cherry and greengage. Most of the Botryosphaeriaceae isolates were obtained from wedge-shaped necrosis (39.3%) followed by central necrosis (19.7%), black spots (13.1%), irregular-shaped necrosis (11.5%), brown to black streaking (11.5%) and arch-shaped necrosis (4.9%). Two species, *N. parvum* and *D. seriata*, were associated with all kinds of wood lesions, while *L. theobromae* and *B. dothidea* were only obtained from wedge-shaped necrosis and central necrosis. *Spencermartinsia viticola* was only isolated from wedge-shaped necrosis and brown to black streaking. The isolation of more than one fungal species from a single wood lesion type occurred only three times, i.e. (i) *D. seriata* and *N. parvum* were isolated from one sample of apricot with wedge-shaped necrosis in Kerman, (ii) *P. minimum* and *P. parasiticum* were obtained from one sample of peach with irregular-shaped necrosis in Fars and (iii) *N. parvum*, *P. minimum* and *Paecilomyces* sp. were isolated from a single symptomatic sample of apricot with central necrosis in Kerman.

The BT sequences of the *Phaeoacremonium* isolates from this study had 99-100% identity with isolates previously identified as *P. minimum* [strain STE-U 6088,

GenBank accession No. EU128062 (Damm *et al.*, 2008)], *P. parasiticum* [strain CBS 109665, AY328378 (Baddley *et al.*, 2006)], *P. viticola* [strain STE-U 5965, EU128093 (Damm *et al.*, 2008)], *P. tuscanum* [strain 1PaI, EU863458 (Essakhi *et al.*, 2008)] and *P. krajdenui* [strain CBS 110368, AY579332 (Mostert *et al.*, 2005)]. BLASTn searches in GenBank showed that ITS sequences of *Botryosphaeriaceae* isolates had 99-100% identity with isolates previously described as *N. parvum* [strain CBS110301, AY259098 (Alves *et al.*, 2004)], *B. dothidea* [strain CBS 116741, AY640254 (Phillips *et al.*, 2005)], *D. mutila* [strain CBS 112553, AY259093 (Alves *et al.*, 2004)], *L. theobromae* [strain CBS190.73, EF622068 (Alves *et al.*, 2008)], *S. viticola* [strain CBS 117009, KF766228 (Slippers *et al.*, 2013)] and *D. seriata* [strain CBS119049, DQ458889 (Alves *et al.*, 2006)]. The EF sequences of these isolates were 99-100% identical to that of *N. parvum* [strain CBS 110301, AY573221 (Phillips *et al.*, 2005)], *B. dothidea* [strain CBS 110302, AY573218 (Phillips *et al.*, 2005)], *D. mutila* [strain CBS 112553, AY573219, (Phillips *et al.*, 2005)], *L. theobromae* [strain CBS190.73, EF622048 (Alves *et al.*, 2008)], *S. viticola* [strain CBS 117006, AY905562 (Luque *et al.*, 2005)] and *D. seriata* [strain CBS 112555, AY573220 (Phillips *et al.*, 2005)]. In addition to these fungal genera, some *Phaeoacremonium* spp. (from black spots, central necrosis, irregular-shaped necrosis and wedge-shaped necrosis), *Phoma* spp. (from black spots, irregular-shaped necrosis and wedge-shaped necrosis), *Phomopsis* spp. (from wedge-shaped necrosis), *Paecilomyces* spp. (from all wood lesions, with the exception of arch-shaped necrosis) and *Natrassia mangiferae* (from irregular-shaped necrosis, wedge-shaped necrosis and central necrosis) were also isolated from stone fruit trees, but were not considered in this study.

**Pathogenicity tests.** Thirty days post inoculation, all species produced wood discoloration that appeared as brownish to black necrotic areas extending above and below the inoculation point (Fig. 2). After cross sectioning the inoculated shoots, it was determined that *Phaeoacremonium* spp. produced small irregular-shaped necrosis while the *Botryosphaeriaceae* spp. produced a wedge-shaped necrosis. The results of the pathogenicity tests (Tables 2 and 3) showed a variation of lesion lengths and re-isolation frequencies of inoculated fungi on hosts. On peach, the most virulent species were *N. parvum* (43.2 mm) and *P. parasiticum* (38.5 mm) while lesions caused by *D. seriata* (11.8 mm) were not significantly different from the negative control (7.2 mm). Re-isolation percentages were between 58.3% (*D. seriata*) and 100% (*B. dothidea* and *P. parasiticum*) on this host. On apricot, two species, *N. parvum* (44.6 mm) and *P. parasiticum* (38.5 mm), produced the longest lesions and were the most virulent species. *Spenceriartinsia viticola* (17.3 mm) produced the shortest lesions than the other *Botryosphaeriaceae* species on apricot but still differed significantly from the control (8.3 mm). There was no significant difference between length of the



**Fig. 2.** Pathogenicity tests of *Phaeoacremonium* and *Botryosphaeriaceae* species on detached shoots of *Prunus* spp. 30 days post inoculation. a: control (cherry); b: lesions caused by *D. seriata* on cherry; c: *P. parasiticum* on cherry; d: *L. theobromae* on cherry; e: *P. minimum* on cherry; f: *P. minimum* on sour cherry; g: *P. viticola* on sour cherry; h: *N. parvum* on cherry; i: *P. tuscanum* on cherry; j: control (sour cherry); k: *P. parasiticum* on greengage; l: *P. minimum* on peach; m: *N. parvum* on peach; n: *N. parvum* on greengage; o: control (apricot); p: *B. dothidea* on apricot; q: *L. theobromae* on apricot; r: *B. dothidea* on cherry (white arrows show the point of inoculation and red brackets indicate lesions length). (Scale bar = 5 mm).

lesions caused by *P. viticola* and the negative controls on apricot. Inoculated isolates were re-isolated from this host with frequencies of 58.3% (*P. viticola* and *D. seriata*) to 100% (*N. parvum*, *B. dothidea* and *P. minimum*).

*Neofusicoccum parvum* (36.2 mm) and *S. viticola* (12.0 mm) produced the longest and shortest necrotic lesions on cherry, respectively. *Phaeoacremonium krajdenui* produced smaller lesions (13.2 mm) than the other species of *Phaeoacremonium* on cherry but this species could also be considered pathogenic, since the lesion lengths were significantly longer than the negative controls (6.8 mm). *P. parasiticum* had a re-isolation rate of 100%, while the remaining species were re-isolated at frequencies between 33.3% to 91.7% on cherry. *N. parvum* (39.5 mm) and *P. parasiticum* (34.3 mm) were again the fungi that produced the longest lesions and were the most virulent species on detached shoots of sour cherry. *Diplodia seriata* (9.2 mm) and *P. krajdenui* (12.6 mm) did not induce significant lesions compared to controls (6.8 mm) on this host. Inoculated

**Table 2.** Mean lesion length caused by *Phaeoacremonium* species on detached shoots of *Prunus* spp. and re-isolation frequencies from observed lesions.

Isolates				Mean lesion length (mm)**					Re-isolation frequency %				
Species	KER-U no.*	GenBank Accession numbers	Original host	Peach	Apricot	Cherry	Sour cherry	Green-gage	Peach	Apricot	Cherry	Sour cherry	Green-gage
<i>Phaeoacremonium parasiticum</i>	PRRCEa	KJ561167	sour cherry	38.5 a	46.5 a	32.2 a	34.3 a	31.2 a	100	83.3	100	83.3	100
<i>Phaeoacremonium minimum</i>	PALCPEC1	KJ561168	peach	29.0 b	28.2 b	21.8 b	21.2 b	28.5 a	91.7	100	83.3	41.7	83.3
<i>Phaeoacremonium tuscanum</i>	PTPECb	KJ561169	peach	27.8 bc	25.8 b	22.5 b	20.7 b	21.3 b	83.3	91.7	66.7	33.3	83.3
<i>Phaeoacremonium krajdenui</i>	PARAPR2	KM433842	apricot	15.2 d	18.3 c	13.2 d	12.6 c	9.0 c	66.7	83.3	33.3	66.7	91.7
<i>Phaeoacremonium viticola</i>	PMVCHER1	KJ561166	cherry	23.8 c	11.2 d	17.3 c	19.7 b	19.7 b	83.3	58.3	58.3	41.7	66.7
Agar plug				7.5 e	8.3 d	6.8 e	6.8 c	8.0 c	0	0	0	0	0
LSD				4.3	3.5	3.9	6.1	3.8					

\* Culture collection of Plant Protection Department, College of Agriculture, Shahid Bahonar University of Kerman, Kerman, Iran.

\*\* Lesions lengths followed by the same letter were not significantly different ( $P < 0.01$ ).

fungi were re-isolated with frequencies between 33.3% (*P. tuscanum*) and 100% (*L. theobromae*) from the inoculated shoots of sour cherry. On detached shoots of greengage, *N. parvum* produced the most extensive lesions (38.8 mm), although they did not differ significantly from those of *L. theobromae* (36.3 mm). The mean lesion length from *P. parasiticum* (31.2 mm) was significantly longer than those of the other *Phaeoacremonium* spp. on greengage, but it did not differ significantly from *P. minimum* (28.5 mm). *Spencermartinsia viticola* (10.2 mm), *D. seriata* (11.0 mm) and *P. krajdenui* (9.0 mm) were the Botryosphaeriaceae and *Phaeoacremonium* species that did not differ from control (8.0 mm) and could, therefore, be regarded as non-pathogens. Re-isolation of the inoculated fungi was successful in the range of 41.7% (*D. mutila*) to 100% (*P. parasiticum*) on greengage shoots. No *Phaeoacremonium* or Botryosphaeriaceae fungi were re-isolated from controls.

## DISCUSSION

Results of field surveys confirmed the occurrence of various external disease symptoms on stone fruit trees in Iran and six internal symptom types in wood, including wedge-shaped necrosis, irregular-shaped necrosis, central wood necrosis, arch-shaped necrosis, black spots and brown to black wood streaking. The internal wood symptoms were similar to those described by various authors for trunk diseases of grapevine, pome fruit trees and ornamental trees (Péros *et al.*, 2008; Van Niekerk *et al.*, 2011; Cloete *et al.*, 2011; Sami *et al.*, 2014; Mohammadi *et al.*, 2014; Hashemi and Mohammadi, 2016; Mohammadi and Sharifi, 2016).

Five *Phaeoacremonium* species were obtained during this study, among which *P. minimum* accounted for 54.7% of total *Phaeoacremonium* isolates. *Phaeoacremonium minimum* was also the most common species of this genus associated with grapevine Petri disease (Mostert *et al.*, 2006;

Essakhi *et al.*, 2008). This species has already been reported in Iran from grapevine showing yellowing and reduced growth plus different symptoms in the wood, including black spot and central necrosis (Mohammadi *et al.*, 2013a), date palm (Mohammadi, 2014), cypress (Mohammadi *et al.*, 2014), apple, pear, quince (Sami *et al.*, 2014) and walnut trees (Mohammadi *et al.*, 2013c).

In the present study, *Phaeoacremonium* species were mostly recovered from central necrosis. In particular, *P. minimum* was isolated from all internal wood lesions types while *P. viticola* and *P. krajdenui* were only obtained from brown to black streaking. *Phaeoacremonium* species were isolated from different kinds of lesions associated with trunk diseases of various plants. Péros *et al.* (2008) isolated *P. minimum* more frequently from central necrosis of grapevine in France. On the contrary, Berraf-Tebbal *et al.* (2011) and Van Niekerk *et al.* (2011) found *Phaeoacremonium* species commonly associated with wedge-shaped necrotic symptoms on grapevine in Algeria and South Africa, respectively. In a study conducted by Cloete *et al.* (2011), *P. minimum*, *P. iranianum*, *P. mortoniae* and *P. viticola* were mostly isolated from wedge-shaped and brown internal necrotic lesions on pome fruit trees in South Africa. Hashemi and Mohammadi (2016) and Moreno-Sanz *et al.* (2013) recorded high isolation percentages of *Phaeoacremonium* spp. from black spots on willow and grapevine in Iran and Northern Spain, respectively. In our work *P. minimum* and *P. parasiticum* were isolated from apricot, peach, cherry and sour cherry, while *P. viticola* and *P. tuscanum* were isolated only from cherry and peach trees, respectively. *P. africanum*, *P. minimum*, *P. iranianum*, *P. parasiticum*, *P. pallidum*, *P. scolyti*, *P. venezuelense* and *P. griseo-olivaceum* were previously reported on apricot (Howksworth *et al.*, 1976; Damm *et al.*, 2008; Olmo *et al.*, 2014; Arzanlou *et al.*, 2014). Of the different *Phaeoacremonium* species, only *P. minimum* and *P. scolyti* have previously been reported from peach trees (Damm *et al.*, 2008); therefore this study is the first report of *P. tuscanum* on this woody host. Based

**Table 3.** Mean lesion length caused by Botryosphaeriaceae species on detached shoots of *Prunus* spp. and re-isolation frequencies from observed lesions.

Species	Isolates				Mean lesion length (mm) **					Re-isolation frequency %				
	KER-U no.*	GenBank Accession numbers		Original host	Peach	Apricot	Cherry	Sour cherry	Green-gage	Peach	Apricot	Cherry	Sour cherry	Green-gage
		ITS	EF1- $\alpha$											
<i>Neofusicoccum parvum</i>	NPPEC1	KF535912	KP793705	peach	43.2 a	44.6 a	36.2 a	39.5 a	38.8 a	91.7	100	91.7	91.7	83.8
<i>Botryosphaeria dothidea</i>	BDAPR1	KF535900	KP793701	apricot	20.3 c	30.0 c	27.2 b	16.2 c	23.0 b	100	100	91.7	66.7	83.8
<i>Diplodia mutila</i>	DMAPR1	KF535901	KP793702	peach	18.8 cd	24.0 d	18.5 d	17.0 c	19.2 c	83.3	83.3	58.3	58.3	41.7
<i>Lasiodiplodia theobromae</i>	LTAPR1	KF535909	KP793703	apricot	35.5 b	39.8 b	23.1 c	32.7 b	36.3 a	83.3	91.7	33.3	100	91.7
<i>Spenceriartinsia viticola</i>	BVPEC1	KJ561170	KP793706	peach	14.8 de	17.3 e	12.0 e	12.7 d	10.2 d	91.7	66.7	83.3	83.3	58.3
<i>Diplodia seriata</i>	DSCHR1	KF535905	KP793704	cherry	11.8 ef	29.2 c	19.8 cd	9.2 e	11.0 d	58.3	58.3	33.3	83.3	83.8
Agar plug					7.5 f	8.3 f	6.8 f	6.8 e	8.0 d	0	0	0	0	0
LSD					5.1	2.5	3.7	2.9	3.6					

\* Culture collection of Plant Protection Department, College of Agriculture, Shahid Bahonar University of Kerman, Kerman, Iran.

\*\* Lesions lengths followed by the same letter were not significantly different ( $P < 0.01$ ).

on previous studies, *P. parasiticum* and *P. minimum* were found affecting cherry trees in Greece (Rumbos, 1986) and Iran (Mousavi *et al.*, 2014), respectively. In this study, *P. parasiticum*, *P. viticola* and *P. minimum* were isolated from cherry trees showing decline symptoms. Although there is no report of *Phaeoacremonium* species on sour cherry, in the current study two species of this genus, *P. minimum* and *P. parasiticum*, were recovered from this woody host. During this study, only one isolate of *P. krajdenui* was obtained from an apricot tree showing dieback and gummosis and brown to black streaking in cross section. This species was reported as a pathogen of grapevines in South Africa and Spain (Mostert *et al.*, 2005; Gramaje *et al.*, 2011). *P. krajdenui* has not been reported from Iran; therefore this is the first report of this species in this country.

Generally we found members of the Botryosphaeriaceae predominantly in wedge-shaped necrosis. Botryosphaeriaceae species were the most abundant fungi isolated from this kind of wood lesion on grapevine (Mugnai *et al.*, 1999; Armengol *et al.*, 2001; Úrbez-Torres *et al.*, 2006; Luque *et al.*, 2009; Van Niekerk *et al.*, 2011), date palm (Mohammadi, 2014), cypress (Mohammadi *et al.*, 2014) and pome fruit trees (Cloete *et al.*, 2011). Of the six Botryosphaeriaceae species encountered in this study, *N. parvum* and *D. seriata* were isolated from all kind of wood lesions while the other four species were only obtained from two or three kinds of recorded wood lesions. *Diplodia seriata* was also isolated from various wood lesions of grapevines in the east of France (Kuntzmann *et al.*, 2010). Hashemi and Mohammadi (2016) recently reported *D. seriata* occurring on wedge-shaped necrosis, irregular wood necrosis, central necrosis and black spots of willow trees in Iran. In accordance with previous studies (Luque *et al.*, 2009; Cloete *et al.*, 2011; Moreno-Sanz *et al.*, 2013; Mohammadi,

2014; Mohammadi *et al.*, 2014; Hashemi and Mohammadi, 2016), our results indicate that the type of internal wood lesions of stone fruit trees is not linked to either the external disease symptoms or to the causative fungi.

The majority of Botryosphaeriaceae isolates were recovered from peach and apricot (24 and 20 isolates, respectively). *Diplodia seriata* comprised 31.1% of the Botryosphaeriaceae isolates, and was recovered from all tree species. *Diplodia seriata* has previously been reported affecting *P. salicina*, *P. persica*, *P. armeniaca* and *P. persica* var. *nucipersica* in South Africa (Damm *et al.*, 2007; Slippers *et al.*, 2007) and *P. dulcis* in USA (Inderbitzin *et al.*, 2010). *Diplodia seriata* is common on peach worldwide (Britton and Hendrix, 1982) and has been found on apricot in USA (Smith and Stanosz, 2006). In Iran, however, this is its first report on cherry, sour cherry and greengage.

In the current study, 17 isolates of *N. parvum* were isolated from peach, apricot and cherry trees. This fungus was the only Botryosphaeriaceae species present in all provinces surveyed. Since in previous studies *N. parvum* was reported on *P. dulcis* (Inderbitzin *et al.*, 2010; Gramaje *et al.*, 2012), *P. persica* (Cunnington *et al.*, 2007), *P. armeniaca* (Gramaje *et al.*, 2012) and *P. avium* (Abdollahzadeh *et al.*, 2013), peach and apricot trees are now new hosts for this pathogen.

*Botryosphaeria dothidea* was isolated from apricot and cherry trees. This species was reported from *P. dulcis* in USA (Inderbitzin *et al.*, 2010) and Spain (Gramaje *et al.*, 2012) and *P. persica* in Iran (Abdollahzadeh *et al.*, 2013). *Botryosphaeria dothidea* was isolated for the first time from necrotic wood of apricot and cherry trees. Nine isolates of *D. mutila* were obtained from peach, cherry and apricot trees. This species has been reported from peach (Laundon, 1973; Sutton, 1980) and apricot (Sutton, 1980)

elsewhere in the world and this is the first reported occurrence of this species on cherry. In our work, *L. theobromae* was obtained only from apricot and greengage. In a study conducted by Pusey *et al.* (1995) this species was reported on *P. persica*. Therefore, this study represents the first record of this species on apricot and greengage. *Spenceriaria viticola* was isolated from affected wood of peach, cherry and greengage. This species has previously been reported from *P. persica* var. *nucipersica* and *P. salicina* in South Africa (Damm *et al.*, 2007). Therefore, this study is the first reported occurrence of *S. viticola* on cherry and greengage trees.

Generally, no relationship was found between the expression of external disease symptoms and any specific wood lesion type or fungal species on stone fruit trees, which is in accordance with previous studies on grapevine in France (Péros *et al.*, 2008) and Spain (Luque *et al.*, 2009) and ornamental trees such as cypress (Mohammadi *et al.*, 2014) and willow in Iran (Hashemi and Mohammadi, 2016). Fungal trunk diseases are considered as complex trunk abnormalities in the sense that many fungi concur to produce the overall external and internal symptoms. Therefore more than one fungal taxon can be obtained from one type of wood lesion as it was reported in the last decades on grapevine from different countries (Péros *et al.*, 2008; Luque *et al.*, 2009; White *et al.*, 2011; Moreno-Sanz *et al.*, 2013).

Pathogenicity tests showed a variation in lesion lengths between fungal species and inoculated hosts. *Neofusicoccum parvum* was the most aggressive species of Botryosphaeriaceae on all inoculated hosts. This species was reported to be the most pathogenic species on grapevine in South Africa (Van Niekerk *et al.*, 2004), Spain (Luque *et al.*, 2007), Eastern Australia (Savocchia *et al.*, 2007) and California (Úrbez-Torres and Gubler, 2009).

The results of our study confirmed that *D. seriata* was pathogenic to apricot and cherry and non-pathogenic to peach, sour cherry and greengage. There are conflicting reports about the pathogenicity of *D. seriata* in different studies. This species was reported to be the cause of black dead arm of grapevine in Chile (Auger *et al.*, 2004) and Lebanon (Choueiri *et al.*, 2006). Based on pathogenicity tests, *D. seriata* was considered as a virulent species on grapevine in South Africa (Van Niekerk *et al.*, 2004) and Eastern Australia (Savocchia *et al.*, 2007), and on detached shoots of *Prunus* (Damm *et al.*, 2007) and pome fruit trees (Cloete *et al.*, 2011) in South Africa. This species was considered intermediately virulent on grapevine in California (Úrbez-Torres and Gubler, 2009) and weakly pathogenic on this host in Portugal (Phillips, 2002), USA (Úrbez-Torres and Gubler, 2009), Spain (Luque *et al.*, 2009) and Mexico (Úrbez-Torres *et al.*, 2008). In another study, *D. seriata* did not cause any symptom on inoculated grapevine cuttings in Western Australia (Taylor *et al.*, 2005) and detached green shoots of grapevine in South Africa (Van Niekerk *et al.*, 2004). Several reasons can explain these differences in virulence, including inoculation methods,

differences in cultivar susceptibility, incubation periods, type of inoculum used, age of the host, type of inoculated tissue and origin of the isolates (Úrbez-Torres *et al.*, 2008; Úrbez-Torres, 2011).

Of the five *Phaeoacremonium* species found during this study, *P. parasiticum* caused the longest lesions compared to the other species on all of the hosts tested but there was no significant difference between the length of lesions caused by *P. parasiticum* and *P. minimum* on greengage. These results are consistent with previous pathogenicity trials conducted in South Africa, in which *P. parasiticum* was shown to be one of the most virulent *Phaeoacremonium* species tested on detached green plum and apricot shoots (Damm *et al.*, 2008). Rumbos (1986) showed *P. parasiticum* to be pathogenic to some stone fruit trees such as peach, cherry and apricot. Small irregular-shaped necrosis (caused by *Phaeoacremonium* species) and young wedge-shaped necrosis (produced by Botryosphaeriaceae species) were observed when cross sections were made from inoculated detached shoots. These results are consistent with previous pathogenicity trials of *Phaeoacremonium* and Botryosphaeriaceae species on pistachio trees in Iran (Mohammadi *et al.*, 2015). Úrbez-Torres *et al.* (2008) also showed that *D. seriata* and *L. theobromae* cause a wedge-shaped canker on inoculated rooted grapevine cuttings in Mexico.

Our study adds to the current knowledge of fungal pathogens infecting and causing decline of stone fruit trees in Iran. This study also revealed that fungal trunk pathogens that were isolated and reported from grapevines in Iran are also present, capable of infecting stone fruit trees and cause diseases in these woody hosts. *Phaeoacremonium* and Botryosphaeriaceae species that cause grapevine trunk diseases were shown not to be host-specific, and had been also isolated from other woody trees. Recently, special attention has been given to alternative woody hosts that are often planted next to vineyards. Therefore, the presence of these pathogens in stone fruit trees could threaten vineyards planted in close proximity to these woody plants.

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