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Fungal pathogens associated with branch and trunk cankers of nut crops in Iran

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Abstract

Branch and trunk canker diseases have become prevalent on nut crops in Iran. During 2015 to 2018, extensive field surveys were conducted on 58 almond, 43 pistachio and 80 walnut orchards in Iran to study fungal pathogens associated with symptomatic trees. One hundred and fifty-six representative fungal isolates were selected and identified based on morphological characteristics and by phylogenetic comparison of DNA sequence data. Fungal species found were *Collophorina hispanica*, *Pleurostoma richardsiae,* nine species of *Phaeoacremonium* (namely *P. angustius*, *P. cinereum*, *P. italicum*, *P. fraxinopennsylvanicum*, *P. minimum*, *P. parasiticum*, *P. scolyti*, *P. tuscanum* and *P. viticola*), eleven species of Botryosphaeriaceae (namely *Botryosphaeria dothidea*, *Diplodia gallae*, *D. mutila*, *D. seriata*, *Dothiorella plurivora*, *Do. sarmentorum*, *Do. viticola*, *Lasiodiplodia citricola*, *L. mahajangana*, *L. theobromae* and *Neofusicoccum parvum*), four species of Diatrypaceae (namely *Cryptosphaeria pullmanensis*, *Diatrype whitmanensis*, *Eutypella citricola* and *E. vitis*) and two non-identified *Eutypella* spp. (*Eutypella* sp. 1 and *Eutypella* sp. 2). Some of these species represent new reports in Iran and/or are reported for the first time in their respective hosts. Pathogenicity tests demonstrated that most of these fungi were pathogenic to inoculated almond, pistachio and walnut shoots. Therefore, more importance should be given to fungal trunk pathogens in Iran, and specific management strategies should be included within the nut crops IPM management programs, with the aim of improving their sustainability.

Key words: Almond, Botryosphaeriaceae, Diatrypaceae, *Phaeoacremonium*, Pistachio, Walnut

Introduction

Iran is one of the most important nut crops producing countries worlwide, with almonds, pistachios and walnuts of special agricultural importance to the country. Almond (*Prunus dulcis*, Rosaceae) is native to Mediterranean regions of the Middle East. Iran is considered the main center of origin of domestic almonds and one of the richest sources of germplasm diversity (Vezvaei 2003). Iran produces about 111,000 metric tons of almonds with 199,000 ha, being the fourth largest producer of this nut crop in the world (FAO 2018). Pistachio (*Pistacia vera*, Anacardiaceae), originates from Central Asia and the Middle East. Pistachio growing area in Iran is about 457,000 ha, producing 315,000 metric tons, being one of the world leading producers (FAO 2018). Walnut (*Juglans regia*, Juglandaceae) is one of the most traditional nut crops in Iran. This tree is known as the Persian walnut and Iran has been regarded as the region of origin of this species, where it was probably domesticated and then introduced to other regions of the world with temperate climate (McGranahan and Leslie 1991; Bayazit et al. 2007). According to FAO (2018), with 150,000 ha and 405,000 tons of walnut, Iran is the third largest producer of this crop in the world.

Nut crops are affected by many fungal pathogens, which can reduce their productivity dramatically (Teviotdale et al. 2002; Moral et al. 2019). Among them, branch and trunk canker diseases are becoming one of the most important limiting factors for nut crops production worldwide (Gramaje et al. 2016). Fungal species belonging to the genera *Collophorina*, *Pleurostoma* and *Phaeoacremonium*, and members of the Botryosphaeriaceae and Diatrypaceae families have been reported associated with branch and trunk canker diseases on almond, pistachio and walnut crops (Slippers et al. 2007; Trouillas et al. 2010a; Gramaje et al. 2012).

The genus *Collophorina*, was introduced by Damm et al. (2010) with five species, namely *C. africana*, *C. capensis*, *C. paarla*, *C. pallida* and *C. rubra*. These species were recovered from necrotic wood lesions of peach and nectarine trees in South Africa. *Collophorina hispanica* was reported causing wood cankers of almonds in Spain (Gramaje et al. 2012) and Iran (Arzanlou et al. 2016), and this fungus, together with *C. paarla*, was also isolated from branch cankers of almond in California (Holland et al. 2018).

Pleurostoma richardsiae (formerly *Phialophora richardsiae*) was previously reported as a human pathogen but, recently, the fungus has also been isolated from plants associated with esca and Petri disease of grapevine in California and Italy (Rolshausen et al. 2010; Carlucci et al. 2015a). A study conducted in Spain revealed that *P. richardsiae* was highly pathogenic to almond (Olmo et al. 2015).

Species of *Phaeoacremonium* are well known plant pathogens causing wilting and dieback of woody plants (Mostert et al. 2006; White et al. 2011; Gramaje et al. 2015). In Spain, *P. amygdalinum* and *P. iranianum* were isolated from almond trees showing internal necrosis and brown to black vascular streaking (Gramaje et al. 2012), and *P. minimum* was

found associated with the decline of almond trees in nursery conditions (Marín-Terrazas et al. 2016). During a survey on stone fruit trees in Iran, *P. minimum* was also isolated from affected almond trees in Kerman province (Mousavi et al. 2014). In a study conducted by Mohammadi et al. (2015) in Iran, four species of *Phaeoacremonium*, *P. cinereum*, *P. minimum*, *P. parasiticum* and *P. viticola* were isolated from pistachio trees showing decline symptoms. In 2018, *P. sicilianum* was reported from *Juglans* sp. in South Africa (Spies et al. 2018). Recently, this species has been isolated from walnut trees in The Czech Republic (Eichmeier et al. 2019). In Iran, a survey conducted by Mohammadi et al. (2013) in Ardabil province (northwest of Iran), identified of two species of this genus, *P. cinereum* and *P. minimum* from walnut trees showing dieback symptoms.

The family Botryosphaeriaceae contains many fungal species, which are considered saprophytes, endophytes or plants pathogens associated with a wide range of fruit, ornamental and forest trees (Phillips et al. 2013). On almond trees, band canker caused by *Botryosphaeria dothidea* was first reported in California in 1959 (English et al. 1966). In South Africa, Slippers et al. (2007) reported *Neofusicoccum australe* as a pathogen of almond trees. In USA, some species such as *B. dothidea*, *Diplodia seriata*, *Dothiorella sarmentorum*, *Macrophomina phaseolina*, *N. mediterraneum*, *N. nonquaestium* and *N. parvum* were reported from almond trees with different internal and external trunk canker disease symptoms (Inderbitzin et al. 2010). Subsequent surveys conducted on almond trees in California showed that *Lasiodiplodia theobromae* (Chen et al. 2013b), *Do. iberica* (Doll et al. 2015) and *Neoscytalidium dimidiatum* (Nouri *et al*. 2018) are also pathogens associated with branch cankers. In Spain, several Botryopshaeriaceae species such as *B. dothidea*, *D. olivarum, D. seriata, N. luteum*, *N. mediterraneum* and *N. parvum* have been reported from almond trees (Gramaje et al. 2012; Olmo et al. 2016). A panicle and shoot blight caused by *B. dothidea* was reported for the first time on pistachio in California in 1991. Shoot blight symptoms included dark brown to black lesions at the base of shoots (Michailides 1991). The fungus *N. mediterraneum* has also been described as a serious threat to the pistachio industry in California (Chen et al. 2014b; Michailides and Morgan 2004). This species had been also reported on pistachio in Spain (Moral et al. 2010), together with *N. australe* (Armengol et al. 2008). In 2014, eight Botryosphaeriaceae species were reported on pistachio trees in California; these included *B. dothidea*, *D. seriata*, *Do. iberica*, *Do. sarmentorum*, *L. citricola*, *L. gilanensis*, *N. mediterraneum* and *N. vitifusiforme*. In this work, *B. dothidea* and *N. parvum* were also reported from pistachio in Greece (Chen et al. 2014b). In another study, *L. americana* and *N. hellenicum* were reported from pistachio trees in USA and Greece, respectively (Chen et al. 2015). According to a study conducted by Mohammadi et al. (2015), *B. dothidea*, *Do. viticola* and *N. parvum* can infect pistachio trees in Iran. Several species of Botryosphaeriaceae including *B. dothidea*, *D. mutila*, *D. seriata*, *Do. iberica*, *L. citricola*, *N. mediterraneum*, *N. nonquasitum N. parvum, N. vitifusiforme* and *Neoscytalidium dimidiatum* have been isolated and reported from walnut trees in California (Inderbitzin et al. 2010; Trouillas et al. 2010b; Chen et al. 2013a, 2014a). *N. parvum* has also been reported from this host plant in China (Yu et al. 2015). Li et al. (2016) reported the occurrence of *B. dothidea* and *L. pseudotheobromae* on English walnut in China.

In a recent survey conducted in The Czech Republic, *D. seriata* and *Do. omnivora* have been reported from English walnut (Eichmeier et al. 2019). Some members of the family Botryosphaeriaceae have also been reported from walnut trees in Iran. These include, *B. dothidea*, *N. parvum* (Abdollahzadeh et al. 2013), *D. seriata* (Mohammadi et al. 2013), and *L. iraniensis* (Abdollahzadeh et al. 2010).

Species of the family Diatrypaceae are predominantly saprotrophic on the decaying wood of trees. However, some species of this family are well known pathogens associated with declining plant hosts (Glawe and Rogers 1984). According to studies on trunk canker diseases conducted in different countries, various species in the family Diatrypaceae, including *Eutypella*, *Eutypa* and *Cryptovalsa* have been found associated with the decay of nut crops (Carter 1982; Gramaje et al. 2012; Rumbos 1984). Almond has been indicated as a host for *Eutypella prunastri* (Ellis and Ellis 1997). *Eutypa lata* is an important plant pathogen in the family Diatrypaceae having a worldwide distribution causing dieback and decline on many tree species worldwide. This species has been already reported on almond (Trouillas and Gubler 2010a; Gramaje et al. 2012; Moyo et al. 2018), pistachio (Rumbos 1986) and walnut trees (Rumbos 1984; Eichmeier et al. 2019). In California, *Cryptovalsa ampelina* has also been reported on walnut trees (Trouillas et al. 2010a). *Eutypella* sp. and *Peroneutypa scoparia* were isolated for the first time from walnut trees in 2019 (Eichmeier et al. 2019). In Iran, *Cryptosphaeria pullmanensis* has been reported from walnut trees (Raoufi et al. 2016).

Previous studies suggested that members of the familiy Botryosphaeriaceae and the genus *Phaeoacremonium* could constitute a threat to various plant species in Iran such as pistachio (Mohammadi et al. 2015), *Prunus* spp. (Abdollahzadeh et al. 2013; Soltaninejad et al. 2017) and pome fruit trees (Sami et al. 2014). However, there is scarce information about the incidence and identity of fungal trunk pathogens, specially Botryosphaeriaceae, Diatrypaceae, *Collophorina, Phaeoacremonium* and *Pleurostoma* species occurring on the main nut crops (almond, pistachio and walnut) cultivated in this country. Therefore, the main objectives of this study were: i) to gain more insight into the occurrence of these taxa on almond, pistachio and walnut trees showing branch cankers and trunk disease symptoms in Iran, and ii) to determine their pathogenicity on these hosts.

Materials and Methods

Field surveys, fungal isolation and morphological characterization

From 2015 to 2018, several field surveys were conducted in nut tree (almond, pistachio and walnut) orchards in Fars, Kerman, Kohgiloiyeh and Boirahmad, Isfahan and Yazd provinces in Iran to study fungal pathogens associated with branch and trunk canker diseases. Wood samples were collected from trunk and branches of trees showing cankers and dieback symptoms. Samples were also collected from pruning wood debris left in the orchards and inspected for the presence of fruiting bodies (pycnidia or ascocarps) on the bark surface. In this study 58 almond, 43 pistachio and 80 walnut orchards were visited. Wood samples were collected from 815 symptomatic trees including 294 almond, 181

pistachio and 331 walnut trees. In total, 842 samples were obtained: almond (305 branches, 36.2% total samples), pistachio (197 branches, 23.4%) and walnut (340 branches, 40.4%). One hundred and fifty wood pruning debris samples containing fruiting bodies were also collected, including 50 almond, 30 pistachio and 70 walnut wood fragments.

Fungal isolations were made by plating out small pieces of discolored wood tissue (4–5 mm) on potato dextrose agar (PDA, Merck, Germany) or malt extract agar (MEA, Merck, Germany) after surface sterilization. Wood chips were sterilised by placing them in 5 % sodium hypochlorite for 2 min and then rinsed in sterile water for 3 min. Isolation of fungi from wood debris was carried out by direct transfer of fungal fruiting bodies (pycnidia) on MEA or preparation of suspension from spores in pycnidia and then transferring them on MEA. In all cases, plates were incubated at 25 °C in darkness. Fungal colonies were transferred to PDA and later single-spored or hyphal-tipped in order to obtain pure cultures. All cultures were maintained in the culture collection of the Department of Plant Protection at the Shahid Bahonar Univesity of Kerman, Kerman. Obtained fungal isolates were tentatively identified based on the main morphological (colony and culture appearance) and microscopic characters (conidia, phialides and conidiophores).

Collophorina species were identified by the presence of conidiomata, microcyclic conidiation or endoconidia, additional to conidia formed on hyphae, as well as size and shape of conidia and conidiophores (Damm et al. 2010).

The *Pleurostoma* isolates were morphologically characterised on MEA (20gr malt extraction; 15 gr agar; Oxoid, UK), PDA (Biokar diagnostics, France), and oatmeal agar (OA; 60 g oatmeal; Fluca analytical, USA, 12.5 g agar; Oxoid, UK), incubated at 25 ± 2°C in the dark for 16 d. Morphological identification of these isolates was based on the main characters such as conidiophore, phialides, collarettes and conidial morphology (Vijaykrishna et al. 2004; Carlucci et al. 2015a).

Species of *Phaeoacremonium* were identified based on colony and culture characters and microscopic structures. The isolates were grown on MEA, PDA and OA (Crous et al. 1996) and incubated at 25 °C in darkness. After 8 and 16 days, colony color and appearance, pigment production on the media and conidiophore morphology, phialide type and shape, size of hyphal warts and conidial size and shape were recorded for each isolate.

Botryosphaeriaceae spp. were induced to produce fruiting bodies by plating them on 2 % water agar (Oxoid, UK) containing sterilised pine needles. Cultures were incubated at 23-25°C under near UV light with a 12 h photoperiod for 20-35 days (Phillips et al. 2013). Produced pycnidia were mounted in water and the length and width, shape, colour and septation of the conidia were evaluated and recorded for each isolate.

Colonies belonging to the Diatrypaceae family were identified based on their colony morphology on PDA and conidial size and shape (Glawe and Rogers 1984; Trouillas et al. 2010a, 2011).

DNA extraction, PCR and DNA sequencing

One hundred and fifty-six representative fungal isolates were selected for molecular identification: Almond (n=51), Pistachio (n=19) and Walnut (n=86) (Table S1). Prior to DNA extraction these isolates were grown on PDA for 10-20

days and total genomic DNA was extracted from mycelium and conidia using CTAB method (Doyle and Doyle 1990). All DNA samples were incubated at −17°C until use for PCR amplification. For *Collophorina*, *Pleurostoma*, Botryosphaeriaceae and Diatrypaceae isolates, the internal transcribed spacer 1 and 2 including the intervening 5.8S nrDNA gene (ITS) and for Botryosphaeriaceae and *Collophorina*, a portion of translation elongation factor 1-alpha (*tef-1α*=EF) region were amplified using primer sets ITS1/ITS4 (White et al. 1990) and EF1-728F/EF1-986R (Carbone and Kohn 1999) or EF1-688F/EF1-1251R (Alves et al. 2008), respectively. The amplification and sequencing of a portion of the glyceraldehyde-3-phosphate dehydrogenase (GDPH) of *Collophorina* were done using the primers GDF1 and GDR1 (Templeton et al. 1992). For *Phaeoacremonium* isolates, oligonucleotide primers T1 and Bt2b (Glass and Donaldson 1995) or BTCadF and BTCadR (Travadon et al. 2015) were used to amplify a part of β-tubulin gene (BT). Initial identification of these isolates was also confirmed by analysis of the actin (ACT) sequences amplified using ACT-512F and ACT-783R primers (Carbone and Kohn 1999). The polymerase chain reaction (PCR) was performed in an Applied Biosystems Veriti 96-well Thermal Cycler (Massachusetts, USA). Electrophoresis of the DNA samples and PCR products were performed on a 1.0 % agarose gel (UltraPureTM Agarose, Invitrogen), stained with REALSAFE Nucleic Acid Staining Solution (Durvis S.L., Valencia, Spain). Bands were visualised under ultraviolet light and a 100-bp ladder (GeneRuler 100 bp DNA Ladder, Thermo Scientific, Vilnius, Lithuania) was used for evaluation of the band sizes. PCR products were purified and sequenced by Bioneer Corporation (Daejeon, South Korea) and Macrogen (Madrid, Spain). All sequences were read and edited with Sequencher software v. 1.8 (Gene Codes Corporation, Ann Arbor, MI), and then run through the BLAST (Basic Local Alignment Search Tool, http://blast.ncbi.nlm. nih.gov/Blast.cgi) to determine their basic identity.

Phylogenetic analysis

The sequences were grouped regarding the fungal families, genera or species and sequenced regions, forming different datasets. Five groups of sequence datasets were generated to confirm the identity of *Collophorina hispanica*, *Pleurostoma richardsiae*, *Phaeoacremonium* spp., Botryosphaeriaceae spp. and Diatrypaceae spp. isolates. For each group, individual loci sequences obtained in this study (Table S1) and those references retrieved from Genbank (Table S2, S3, S4 and S5) were aligned using default settings of ClustalW algorithm (Thompson et al. 1994) included within MEGAX software package (Kumar et al. 2018). The alignments were manually checked and improved where necessary. For multilocus analysis of *C. hispanica*, *P. richardsiae*, *Phaeoacremonium* spp., Botryosphaeriaeae spp. and Diatrypaceae spp., the single locus alignments were concatenated using SequenceMatrix 1.8 (Vaidya et al 2011). Phylogenetic analyses for each locus and concatenated datasets were based on Maximum Likelihood (ML) and Maximum Parsimony (MP). The ML analyses were done with the tool RAxML-HPC2 on XSEDE (Stamatakis, 2014) through the CIPRES Science Gateway

V 3.3 (Miller et al. 2010). A nucleotide substitution model GTRGAMMA was used and the supports of the branches were calculated from 1000 bootstrap replicates. Maximum-parsimony analysis was performed in MEGA X (Kumar et al. 2018) with the Tree-Bisection-Reconnection (TBR) algorithm, where gaps were treated as missing data. Measures calculated for parsimony included tree length (TL), consistency index (CI), retention index (RI) and rescaled consistency index (RC). The robustness of the topology was evaluated by 1000 bootstrap replications (Felsenstein, 1985). Trees were visualized using MEGA X (Kumar et al. 2018) or Fig Tree 1.4.3 (http://tree.bio.ed.ac.uk/software/figtree/). All sequences were deposited in GenBank and were listed in Table S1. The alignments were deposited in TreeBASE under the study number S25374.

Preliminary pathogenicity studies

Fifty-six different fungal isolates representing the fungal species obtained from nut tree species in various locations were selected for pathogenicity studies (Tables 1, 2 and 3). Pathogenicity tests were conducted on detached shoots of almond (8-12 years old), pistachio (15-18 years old) and walnut (17-20 years old) trees under control conditions. Shoots were cut in a uniform length (~35 cm in length and 2.5 cm in diameter) and the outer bark of them sprayed with 70% ethanol. After air drying, each shoot was wounded using a 4-mm sterilized cork borer and then a mycelial plug of the same size taken from the margin of fungal colonies on PDA (10–16 days old) was placed into the created wound. All inoculated sites were covered by moist cotton and then protected by wrapping with Parafilm (Pechiney Plastic Packaging, Menasha, USA). Six shoots per each fungal isolate were used and an equal number was also inoculated with 4 mm non-colonized potato dextrose agar plugs as control treatments. Treatment and control shoots were immediately placed into plastic containers, filled with distilled water (2000 ml), covered with a transparent plastic bag and maintained at 25-27 °C. This experiment was arranged in a completely randomized design and repeated once. All inoculated shoots were collected after 40 days of incubation and the extent of wood discoloration in the wood was measured upward and downward from the point of inoculation. Data were checked by means of Shapiro–Wilk and Kolmogorov–Smirnov tests. Data from the pathogenicity assay was subjected to analysis of variance (one-way ANOVA) using SAS v 9.1 (SAS Institute, Cary, North Carolina, USA). LSD (The least significant difference) test was used for comparison of all treatment means at $P < 0.05$. Fifteen small segments of discolored wood tissues were cut from the edges of lesions produced on the test and control shoots and placed on PDA. Re-isolated fungi were identified as described previously, fulfilling Koch's postulates.

Results

Sampling and collection of fungal isolates

The most important external disease symptoms observed on almond, pistachio and walnut trees were yellowing, gumming, branch cankers and dieback. Internal symptoms included central, irregular, watery or v-shaped necrosis, brown to black wood streaking, and black spots, which were observed in cross sections of diseased branches (Fig. 1).

Fruiting bodies of Botryosphaeriaceae and Diatrypaceae isolates were detected on the surface of the wood debris collected in almond, pistachio and walnut orchards.

In total, 156 representative fungal isolates were selected from symptomatic tissues and wood debris of almond, pistachio and walnut trees. Of these, 133 isolates were obtained from internal wood lesions and 23 isolates were obtained from wood debris. Based on their appearance in culture, morphological and microscopic characteristics, isolates were divided into five main groups including *Collophorina* (5 isolates), *Pleurostoma* (10 isolates), *Phaeoacremonium*, (52 isolates), Botryosphaeriaceae (46 isolates: 25 from infected wood tissues and 21 isolates from wood debris), Diatrypaceae (43 isolates; 41 isolates from discolored wood tissues and 2 isolates from wood debris) (Table S6).

Collophorina isolates formed white to reddish cream colonies on PDA. Members of this group had slow growing mycelium, turning red to blood colour with age and produced a red pigment that coloured the colony and surrounding medium. According to these morphological features, all isolates were tentatively identified as *Collophorina hispanica* (Gramaje et al. 2012).

Pleurostoma isolates were characterized by *Phialophora*-like asexual morph and brown to olive-brown colonies on MEA. This group produced two different types of conidia, brown globose conidia, and hyaline, allantoid to cylindrical conidia. Morphological characteristics recorded for these isolates were consistent with the description of *P. richardsiae* (Vijaykrishma et al. 2004).

Phaeoacremonium isolates were characterized by pale to pale brown to reddish brown flat slow-growing cultures on MEA. Isolates of this group had three types of phialides that were different in size and shape. Sporulation was abundant and conidia were hyaline and aseptate. These characters were consistent with the description of genus *Phaeoacremonium* (Crous et al. 1996; Mostert et al. 2006).

Botryosphaeriaceae isolates were characterized by dark green or grey to dark grey fast-growing mycelium on PDA. These isolates produced black and globose fruiting bodies (pycnidia) on pine needles after 16 to 25 days. Conidia were hyaline or pigmented (Phillips et al. 2013; Slippers et al. 2013; Dissanayake et al. 2016). Based on conidial characteristics these isolates were divided to four subgroups. Some isolates formed conidia narrowly fusiform or irregularly fusiform and were tentatively identified as *Neofusicoccum* spp. or *Botryosphaeria* spp. In other isolates the conidia were initially hyaline, aseptate, thick-walled, becoming 1–2-septate and pale transluscent brown after discharge from the pycnidia. These characteristics were consistent with the description of *Diplodia* spp. Conidia in some isolates were initially hyaline, becoming dark brown and one-septate within the pycnidia. These isolates were tentatively identified as *Dothiorella* spp.

Finally, some isolates of this group produced sub-ovoid to ellipsoid ovoid, initially hyaline and aseptate, dark brown 1-septate conidia after discharge from the conidiomata, showing longitudinal striations. All these isolates were tentatively identified as *Lasiodiplodia* spp. (Phillips et al. 2013; Slippers et al. 2013).

Diatrypaceae isolates were characterized by having white to white-cream cottony slow-growing mycelium on PDA which gradually darkened in the center. Isolates of this group formed small pycnidia on pine needles under continuous fluorescent light after 3-4 weeks. Conidia were filiform and mostly curved in shape, which these morphological features correspond to descriptions of species in the Diatrypaceae family (Glawe and Rogers 1984).

Molecular characterization and phylogenetic analyses

Molecular characterization allowed to confirm the identity of the 156 isolates included in this study. Regarding *Collophorina* and *Pleurostoma* isolates, all of them belonged to the same species: *C. hispanica* and *P. richardsiae,* respectively. The tree topologies of the phylogenies were overall highly concordant between ML and MP inferences. The MP trees are presented, with ML and MP support values at branches for *Phaeoacremonium*, Botryosphaeriaceae and Diatrypaceae.

For *Collophorina* analysis, the ITS region was sequenced from all isolates, and fragments of EF and GAPDH were sequenced from some of them. The concatenated alignment consisted of 21 isolates (5 Iranian isolates, 15 references and 1 outgroup) and 925 characters including gaps (EF: 302, ITS: 480 and GAPDH: 143). The MP analysis resulted in 8 equally most parsimonious trees (TL=527, CI=0.872, RI=0.918, RC=0.8). All Iranian isolates were grouped in the same clade with the *C. hispanica* ex-type, with 100% of bootstrap (Fig. 2).

For *Pleurostoma* analysis, the ITS region was sequenced in all isolates but one (IRNKPH210) (n=9), while BT sequence was generated from representative isolates (n=5). The ACT sequence was generated only from IRNKPH210. The two individual phylogenetic analysis (ITS and BT) resulted in similar tree topology (data not shown). The combined alignment of the Iranian isolates with both sequences (n=5), four sequences of reference isolates and *P. fraxinopensilvanicum* CBS101585 as outgroup, consisted of 1237 characters including gaps (ITS: 514 and BT: 723). The MP analysis resulted in 5 equally most parsimonious trees (TL=737, CI=0.823, RI=0.806, RC=0.663). The Iranian isolates clustered with the reference isolate of *P. richardsiae* (100% bootstrap support) (Fig. 3).

For the *Phaeoacremonium* analysis, the BT sequences were obtained from all *Phaeoacremonium* suspected isolates (n=51), while ACT sequences were obtained from a selection of them (n=33) (Table S1). The concatenated alignment included the isolates with both sequences and consisted of 73 taxa (33 representative Iranian isolates, 39 references and 1 outgroup) with 827 characters (ACT: 226 and BT: 601) including gaps. In the concatenated alignment, 316 characters were conserved and 384 were parsimony informative. The heuristic search resulted in 10 equally most parsimonious trees with TL=1211, CI=0.611, RI=0.916 and RC=0.56. The MP tree showed that Iranian isolates belonged to 9 of 23 clades

of described *Phaeoacemonium* species (Fig. 4). The ITS region of the Iranian *P. cinereum* isolates was also sequenced because in the sequences of the ACT and BT regions, 8 and 6 nucleotide differences were found respectively. The sequence of the ITS region presented 1 difference. These sequence differences did not result into morphological changes that supported the separation of these isolates as a new species. The most frequently species were *P. italicum* (n=18), *P. minimum* (n=12) and *P. parasiticum* (n=8). One or two isolates were identified from the species *P. angustius* (n=2), *P. scolyti* (n=1), *P. tuscanum* (n=1) and *P. fraxinopensilvanicum* (n=1).

For the Botryosphaeriaceae analysis, the ITS and EF sequences were generated for 46 isolates and aligned with 50 reference sequences and three outgroups. The combined alignment consisted of 942 characters including gaps (ITS: 588 and EF: 354). Of these, 596 were constant and 333 parsimony informative. Maximum parsimony analysis resulted in 3 equally most parsimonious trees (TL=715, CI=0.729; RI=0.963, RC=0.702). The showed MP tree revealed 36 well-supported clades corresponding to established species (Fig. 5). Our isolates belonged to 12 of them, being *Diplodia* the dominant genus (37%), followed by *Botryosphaeria* (26%) and *Lasiodiplodia* (20%), being *Botryosphaeria dothidea* (n=12), *D. seriata* (n=10) and *L. theobromae* (n=7) were the principal species isolated. *Diplodia mutila* (n=4), *D. gallae* (n=3), *L. citricola* (n=1), *L. mahajangana* (n=1), *N. parvum*, *Do. sarmentorum* (n=2), *Do. viticola* (n=2), and *Do. plurivora* (n=2) were the other species identified.

For the Diatrypaceae analysis, the ITS sequences of 43 suspected Diatrypaceous isolates were aligned with 27 sequences of reference isolates and two of *Cryptovalsa ampelina* (CBS117485 and DMO100) were used as outgroup. The alignment consisted of 538 characters (including gaps), of which 350 were constant and 177 parsimony informative. Maximum parsimony analysis resulted in 7 equally most parsimonious trees (TL=382, CI=0.661; RI=0.924, RC=0.611). Analysis of the ITS region clearly separated Iranian isolates in 6 clades, 4 of them belonged to the previous described species *Eutypella vitis* (n=4), *E. citricola* (n=2), *Diatrype whitmanensis* (n=1) and *Cryptosphaeria pullmanensis* (n=10). The remaining isolates (n=26) formed 2 well supported clades named here as *Eutypella* sp. 1 (n=23) and *Eutypella* sp. 2 (n=3). *Eutypella* sp. 1 was closely related to *E. cerviculata*, *E. semicircularis* and *Anthostoma decipiens* with 90.8, 90.6 and 91.3 percentage of identities respectively. *Eutypella* sp. 2 was closely related to *E. citricola* and *E. vitis* with 95.7 and 94.7 percentage of identities respectively (Fig. 6).

Pathogenicity tests

Mean lengths of the extent of wood discolorations caused by inoculated isolates on almond, pistachio and walnut branches are shown in Tables 1, 2 and 3, respectively. Based on our results, all inoculated isolates produced brown to dark wood discoloration upward and downward from the point of inoculation after 40 days of incubation (some isolates shown in Fig. 7).

On almond shoots, the longest lesions were caused by isolate IRNBS8 (*D. gallae*), although there was no significant difference between length of lesions produced by this isolate and the isolates: IRNBS54 (*L. theobromae*) and IRNBS30 (*N. parvum*). Isolates IRNM32-2 (*Eutypella* sp. 1) and IRNEU1 (*D. whitmanensis*) resulted in the smallest lesions on this host and were not significantly different from those of the control treatments. Re-isolation percentages of fungi inoculated on almond shoots ranged between 43% (*D. seriata*, isolate IRNBS15) and 86% (*P. minimum*, isolate IRNKPH178).

On pistachio shoots all fungal isolates produced wood lesions statistically different to those caused by the controls. *Eutypella* sp. 2 (isolate IRNM1, 108.4 mm) and one isolate of *B. dothidea* (isolate IRNBS61, 96.0 mm) were the most aggressive isolates, while *E. citricola* (IRNM4) gave the shortest lesions (34.4 mm) on detached shoots of this host. Re-isolation of the inoculated isolates ranged from 43% (*D. seriata*, isolate IRNBS71 and *E. citricola*, IRNM4) to 78% (*Eutypella* sp. 2*,* isolate IRNM1).

On detached shoots of walnut, the isolate IRNKB245 (*L. theobromae*) was the most aggressive, causing the longest (244.4 mm) wood lesions ($P<0.05$), although there were no significant differences among the length of wood lesions produced by this isolate and IRNBS6 (*D. gallae*), IRNBS19 (*B. dothidea*) and IRNM8 (*Eutypella* sp. 2). Isolates IRNKPH176 and IRNKPH209 (*P. italicum*), IRNKB211 (*D. seriata*), IRNKB213 (*B. dothidea*), IRNKB220 (*Do. plurivora*) and IRNKPH177 (*P. cinereum*) were the less aggressive; they produced lesions not significantly different from the controls (11.4 mm). The highest recovery percentage was recorded for *L. theobromae* (93%) and the lowest for *Do. viticola*, *Eutypella* sp. 1, *Eutypella* sp. 2, *P. cinereum* (IRNK72S) (50%). None of the fungal species were re-isolated from the small lesions observed on the control shoots in any of the hosts species inoculated.

Discussion

In recent years, extensive studies have been carried out throughout the world about branch and trunk canker diseases caused by fungal pathogens on various woody plants, including nut trees. However, relatively few of these researches have focused on nut trees in Iran, which is an important world producer of almond, pistachio and walnut. Therefore, this is the most extensive and comprehensive study on these diseases in this country, where the surveys were conducted in five provinces.

The most frequent external disease symptoms were branch cankers and dieback, and cross sections from wood samples revealed a diverse range of internal wood necrosis. Similar internal wood lesion types had been previously described by various authors on different woody plants, such as grapevine (Mostert et al. 2006; White et al. 2011), date palm (Mohammadi 2014), pome fruit trees (Cloete et al. 2011; Sami et al. 2014), pistachio (Mohammadi et al. 2015; Nouri et al. 2019) and some forest and ornamental trees (Hashemi et al. 2017; Kazemzadeh Chakusary et al. 2017). Moreover, wood debris samples examination revealed the presence of Botryosphaeriaceae and Diatrypaceae fruiting bodies.

Based on morphological characters and DNA sequence data, a high diversity of fungal species was found on almond, pistachio and walnut samples. These include *C. hispanica*, *P. richardsiae*, nine *Phaeoacremonium* species (*P. angustius*, *P. cinereum*, *P. italicum*, *P. fraxinopennsylvanicum*, *P. minimum*, *P. parasiticum*, *P. scolyti*, *P. tuscanum* and *P. viticola*), eleven Botryosphaeriaceae species *B. dothidea*, *D. gallae*, *D. mutila*, *D. seriata*, *Do. plurivora*, *Do. sarmentorum*, *Do. viticola*, *L. citricola*, *L. mahajangana*, *L. theobromae* and *N. parvum*, and four species of Diatrypaceae (*C. pullmanensis*, *D. whitmanensis*, *E. citricola* and *E. vitis*) and two non-identified *Eutypella* spp. (*Eutypella* sp. 1 and *Eutypella* sp. 2). In some cases, more than one species or taxon was retrieved from the same plant sample, as previously found by other authors (Gramaje et al. 2012; Sami et al. 2014; Mohammadi et al. 2015; Kazemzadeh Chakusary et al. 2017; Panahandeh et al. 2019).

Collophorina hispanica was isolated only from almond trees. This species was obtained from different internal wood necrosis. Gramaje et al. (2012) characterized *C. hispanica*, as new species from diseased almond trees showing black spots and dark brown to black streaking of the xylem tissues in Spain. This species was reported associated with branch cankers of almond in California (Holland et al. 2018). A survey about fungal trunk pathogens of almond trees conducted in north-west of Iran showed that *C. hispanica* is a dominant species associated with diseased almond trees in this country (Baradaran Bagheri et al. 2015; Arzanlou et al. 2016).

In our work, *P. richardsiae* was isolated from irregular wood necrosis in walnut trees and different internal symptoms in almond samples. Our results are consistent with previous studies in which this species was also recognized as trunk pathogen. Canale et al. (2019) isolated this fungus from olive trees with dark streaking in the inner wood tissues and necrosis symptoms. This pathogen has also been reported from grapevine in South Africa, California, Italy and Spain (Halleen et al. 2007; Rolshausen et al. 2010; Carlucci et al. 2015a; Varela et al. 2016), olive in Italy (Carlucci et al. 2013) and almond in Spain (Olmo et al. 2015). This is the first report of *P. richardsiae* associated with diseased walnut trees in the world.

Phaeoacremonium spp. were one of the most abundant fungal groups isolated from different internal wood necrosis. Nine *Phaeoacremonium* species were isolated from almond, pistachio and walnut trees in this study. These included*, P. angustius*, *P. cinereum*, *P. italicum*, *P. fraxinopennsylvanicum*, *P. minimum*, *P. parasiticum*, *P. scolyti*, *P. tuscanum* and *P. viticola.* Of these, only *P. minimum*, *P. parasiticum* and *P. viticola* were obtained from all the three hosts. *Phaeoacremonium angustius* has been reported from grapevine in Portugal (Chicau et al. 2000), Spain (Garcia-Benavides et al. 2013), apple and grapevine in USA (Groenewald et al. 2001; Rooney-Latham et al. 2005) and quince trees in Germany (Gierl and Fischer 2017). This study is the first report of *P. angustius* from Iran and also the first report of this fungus from almond trees in the world. *Phaeoacremonium cinereum* was isolated only from walnut trees. This species has been previously reported from diseased grapevines in Iran and Spain (Gramaje et al. 2009) and necrotic wood of

walnut trees in Iran (Mohammadi et al. 2013). *Phaeoacremonium fraxinopennsylvanicum* has been identified from different woody hosts such as, black alder in Iran (Kazemzadeh Chakusary et al. 2017), apple in Iran (Sami et al. 2014) and South Africa (Spies et al. 2018) and grapevine in South Africa (White et al. 2011) and Iran (Mohammadi 2011). Our study is the first report of *P. fraxinopennsylvanicum* on walnut trees in the world. *Phaeoacremonium italicum* was the most abundant species in walnut trees. This taxon was also found on almond trees in our surveys. This species was previously detected from quince, common fig, apple, Chinaberry, *Morus* sp., olive, peach, common guava, pomegranate and grapevine (Spice et al. 2018). Our study shows for the first time that almond and walnut wood tissues can be colonized by *P. italicum. Phaeoacremonium minimum* was isolated from almond, pistachio and walnut trees. This fungus was the dominant *Phaeoacremonium* species on almond trees. This species has been previously reported from different host plants such as almond (Mousavi et al. 2014; Marín-Terrazas et al. 2016; Spies et al. 2018), pistachio (Mohammadi et al. 2015), walnut (Mohammadi et al. 2013), ornamental and forest trees (Kazemzadeh Chakusari et al. 2017), apple (Cloete et al. 2011; Arzanlou et al. 2014; Sami et al. 2014) and olive (Úrbez-Torres et al. 2013; Spies et al. 2018). *Phaeoacremonium parasiticum* was the most common species on pistachio trees. This species was also the prevalent species in a previous study about trunk pathogens of pistachio trees in Iran conducted by Mohammadi et al. (2015). This fungus has been retrieved from a wide range of plant hosts (Mostert et al. 2006; Gramaje et al. 2015; Spies et al. 2018). It was previously recorded on cypress (Mohammadi et al. 2014), pome and stone fruit (Sami et al. 2014; Soltaninejad et al. 2017), date palm (Mohammadi 2014) and some forest trees such as Caucasian zelkova, Persian iron wood, Persian poplar, European hornbeam and smooth leaf elm (Kazemzadeh Chakusari et al. 2017) and more recently from Java plum (Panahandeh et al. 2019) in Iran. In this study, only one isolate of *P. scolyti* was obtained from walnut trees. This fungus has previously been reported from persimmon, apple, almond and loquat (Spies et al. 2018), olive (Carlucci et al. 2013; Spies et al. 2018), stone fruit trees (Damm et al. 2008) and grapevine (Gramaje et al. 2008). In Iran, this species has also been isolated from quince and pear (Sami et al. 2014), Persian iron wood and pomegranate (Kazemzadeh Chakusari et al. 2017). Based on literature reviews, this is the first report of *P. scolyti* associated with necrotic wood of walnut trees in the world. *Phaeoacremonium tuscanum* was only found on walnut trees. This species has been reported from grapevine in Italy (Essakhi et al. 2008) and grapevine and peach in Iran (Mohammadi 2012; Soltaninejad et al. 2017). This study also represents the first record of *P. tuscanum* on walnut trees. In our survey, *P. viticola* was isolated from all three nut crops studied. This species was reported affecting grapevine (Mostert et al. 2006; Dupont et al. 2000), kiwifruit in France (Hennion et al. 2001), quince, loquat, common guava, oak, willow and plum trees in South Africa (Damm et al. 2008, Spies et al. 2018) and mountain-ash in Germany (Mostert et al. 2006). In Iran *P. viticola* has been reported from pistachio (Mohammadi et al. 2015) and cherry (Soltaninejad et al. 2017). Therefore, the current study is the first report of *P. viticola* associated with branch canker and dieback on almond and walnut trees worldwide.

Eleven species of Botryosphaeriaceae were recovered from walnut, pistachio and almond trees. These include *B. dothidea*, *D. gallae*, *D. mutila*, *D. seriata*, *Do. plurivora*, *Do. sarmentorum*, *Do. viticola*, *L. citricola*, *L. mahajangana*, *L. theobromae* and *N. parvum.* Of these *B. dothidea* and *D. seriata* were only isolated from all three hosts. Six species, including *B. dothidea*, *D. gallae*, *D. seriata, Do. plurivora, L. theobromae* and *N. parvum* were collected from both affected trees and wood debris, while *Do. sarmentorum* was only obtained from wood debris. Most of them were recovered from walnut (10 species) followed by almond (6 species) and pistachio (2 species). Of the Botryosphaeriaceae species detected in this study, *B. dothidea*, *D. seriata* and *L. theobromae* were the most frequent species. *B. dothidea*, *D. seriata* and *N. parvum* are considered as the most common species associated with grapevine decline in Spain (Armengol et al. 2001, Aroca et al. 2006). *D. seriata* was one of the most frequently isolated Botryosphaeriaceae species in almond in Spain (Olmo et al. 2016), apricot, nectarine, peach and Japanese plum in South Africa (Damm et al. 2007), apple, pear and peach in Uruguay (Sessa et al. 2016) and stone fruit trees in South Africa (Slippers et al. 2007). In Iran, *B. dothidea* has been already reported as the most common Botryosphaeriaceae species in pistachio, and *D. seriata*, *N. parvum* and *B. dothidea* were reported from stone fruit trees (Mohammadi et al. 2015; Soltaninejad et al. 2017). *Botryosphaeria dothidea* was isolated from almond, pistachio and walnut trees. This species causes canker diseases in hundreds of plant species worldwide, including almond and several *Prunus* spp. (English et al. 1966; Inderbitzin et al. 2010; Gramaje et al. 2012), pistachio (Michailides 1991; Chen et al. 2014b), walnut (Li et al. 2016) and grapevine (Van Niekerk et al. 2006; Úrbez-Torres and Gubler 2009). In Iran, this fungus was previously found associated with apricot and sour cherry trees (Soltaninejad et al. 2017), almond (Ershad 2009), pistachio (Mohammadi et al. 2015) and walnut (Abdollahzadeh et al. 2013). Three species of *Diplodia*, *D. seriata*, *D. gallae* and *D. mutila* were found in this study. *D. seriata* has been reported from more than 250 plant hosts in the world. This species has been isolated from almond, pistachio and walnut trees (Inderbitzin et al. 2010; Gramaje et al. 2012; Chen et al. 2014a, b). In Iran, this fungus has previously been reported from walnut and pistachio trees (Mohammadi et al. 2013, 2015). Based on literature reviews, this is the first report of *D. seriata* associated with necrotic wood of almond trees in Iran. Three isolates of *D. gallae* were obtained in this study. Of these, two isolates were collected from wood pruning debris of almond trees and one isolate was also obtained from affected branches of walnut. This species was first described as *Sphaeria gallae* by D. von Schweinitz to name a fungus associated with galls on *Quercus* in the USA. The fungus was subsequently placed in several different genera, namely *Aplosporella*, *Botryodiplodia*, *Macroplodia*, and *Sphaeropsis*. These results suggested that this fungus has dark conidia, which correlate with the strains clustering in *Diplodia* (Yang et al. 2017). This species has been reported as a causal agent of canker of oak trees (Croghan and Robbin 1986). Therefore, almond and walnut trees are reported here as two new woody plant hosts for this fungus. In our survey *D. mutila* was isolated only from almond trees. This pathogen has been reported associated with various forest and fruit trees such as lawson cypress, *Fraxinus* sp., *Populus* sp., English yew (Phillips et al. 2013), walnut trees (Chen et al. 2014a), grapevine (Carlucci et al. 2015b), apple (Úrbez-Torres et al. 2016), date palm

(Mohammadi, 2014) and peach, cherry, apricot (Soltaninejad et al. 2017). However, this is the first report of *D. mutila* on almond trees worldwide. In our survey, three species of *Lasiodiplodia*, namely *L. theobromae*, *L. citricola* and *L. mahajangana* were isolated from almond and walnut trees. Of these, *L. theobromae* was obtained from infected wood tissues of almond and walnut trees as well as wood debris of almond trees. This species has a wide host range and is widely distributed in tropical and subtropical regions (Dissanayake et al. 2016). It has previously been reported as the cause of canker and dieback in many woody plants such as walnut (Haggag et al. 2007), almond, pistachio (Chen et al. 2013b, 2014b), olive (Úrbez-Torres et al. 2013), mango (Abdollahzadeh et al. 2010) and stone fruit trees (Soltaninejad et al. 2017). Our work represents the first report of this species on almond and walnut trees in Iran. Only one isolate of *L. citricola* was obtained from walnut trees. This species was originally described from *Citrus* sp. in Iran (Abdollahzadeh et al. 2010). It has also been documented on peach, pistachio (Chen et al. 2013c, 2014b) and grapevine (Carlucci et al. 2015b). According to Chen et al. (2013a, 2014a), *L. citricola* could cause death of newly grafted walnut trees and infecting walnut trees. Although *L. citricola* has been reported to occur on walnut in other countries, the fungus was found for the first time associated with this nut tree in Iran. *Lasiodiplodia mahajangana* was recovered from walnut trees. This species was first described from Indian almond in Madagascar (Begoude et al. 2011). However, since then this species has been also detected from candelabra and Australian baobab trees in South Africa and Australia, respectively (Van der Linde et al. 2011). More recently, Kazemzadeh Chakusari et al. (2019) isolated and reported this taxon from Caucasian walnut, chestnut-leaved oak, Caucasian zelkova and Smooth leaf elm in the north of Iran. Therefore, walnut tree is reported here as new woody host for this pathogen. In the present study, we isolated three species of *Dothiorella*, namely *Do. plurivora*, *Do. sarmentorum* and *Do. viticola*, from branch samples and wood debris of walnut trees. *Dothiorella plurivora* was recovered from walnut, apple, apricot and grapevine in Spain and *Citrus* sp., Australian pine tree, apple, apricot, *Eucalyptus* sp. and Mediterranean cypress in Southern Iran (Abdollahzadeh et al. 2014). This species has also been isolated from peach and Japanese plum in South Africa (Damm et al. 2008). During a field survey conducted throughout forest areas in the north of Iran, this taxon was also reported from Caucasian persimmon, chestnut-leaved oak, black alder and Persian poplar (Kazemzadeh Chakusary et al. 2019). To our knowledge, this is the first report of *Do. plurivora* from branches and wood debris of walnut trees in Iran. In our work, *Do. sarmentorum* was only detected on the wood debris of walnut trees. *Do. sarmentorum* is a plurivorous species and has been isolated from 34 different host species (Phillips et al. 2013). In Iran, this fungus has been reported from fruit and forest trees such as field elm (Hashemi et al. 2017), quince and also from adult beetles of *Osphranteria coerulescens* (Mohammadi and Sharifi 2016), oak and black alder (Kazemzadeh Chakusari et al. 2019) and walnut (Ershad 2009). Results of this study showed that *Do*. *sarmentorum* can survive and sporulate on wood debris of walnut. In the current work only one isolate of *Do. viticola* was detected from walnut trees showing branch dieback. This fungus has been isolated from different hosts, such as grapevine (Urbez-Torres et al. 2007; Slippers et al. 2013), *Citrus* sp. (Abdollahzadeh et al. 2014; Hamrouni et al. 2018), *Prunus* sp. (Damm et al.

2007; Soltaninejad et al. 2017), and pistachio (Mohammadi et al. 2015). This species has not been detected from walnut trees previously, therefore this is the first record of *Do. viticola* on walnut in the world. *Neofusicoccum parvum* was obtained from diseased walnut trees and wood debris of almond. This cosmopolitan species occurs on many woody plants worldwide. Our findings from wood debris of almond trees allowed the obtention of three isolates of *N. parvum.* This species has been reported in several countries and has also been isolated from various woody plants such as walnut (Inderbitzin et al. 2010; Chen et al. 2014a; Abdollahzadeh et al. 2013), stone fruit trees (Slippers et al. 2007; Inderbitzin et al. 2010; Soltaninejad et al. 2017), Mediterranean cypress (Mohammadi et al. 2014) and pistachio (Chen et al. 2014b; Mohammadi et al. 2015).

In the current study, four species of Diatrypaceae were detected from nut trees, including *C. pullmanensis*, *D. whitmanensis*, *E. citricola* and *E. vitis*. Additionally, two taxa could not be identified to the species level but were closely related to *Eutypella* (*Eutypella* sp. 1 and *Eutypella* sp. 2). Several species in the genera of *Cryptosphaeria*, *Eutypa*, *Cryptovalsa*, *Diatrype* and *Diatrypella* are known to occur on grapevines worldwide (Mostert et al. 2004; Trouillas and Gubler 2004; Pitt et al. 2009; Trouillas et al. 2010a, 2011). *Cryptosphaeria pullmanensis* was only recovered from walnut. This species was first described by Glawe (1984) from fallen branches of western balsam-poplar and then was detected from some woody plants such as grapevine in California (Trouillas et al. 2010a), fremont's cottonwood in USA (Trouillas et al. 2015; Trouillas and Gubler 2016), white willow and white poplar in China (Ma et al. 2016), and walnut in Iran (Raoufi et al. 2016). Only one isolate of *D. whitmanensis* was obtained from almond trees in this study. This species has been reported from big leaf maple, grapevine and California buckeye trees in California (Trouillas et al. 2010a) and elm trees in Iran (Hashemi et al. 2017). This study represents the first record of *D. whitmanensis* on almond in the world. We found two species of *Eutypella*, namely *E. citricola* and *E. vitis* from pistachio and walnut trees, respectively. *Eutypella citricola* has been isolated from *Citrus* spp. in Southern California (Mayorquin et al. 2016), grapefruit, orange, grapevine in Australia (Trouillas et al. 2011) and apricot, Japanese plum in South Africa (Moyo et al. 2018). Based on literature reviews, this is the first time that *E. citricola* has been found on pistachio trees worldwide. In this work, we found *E. vitis* from two sources, affected branches as well as wood debris of walnut trees. In Iran, this species has previously been reported from grapevine (Jordan and Schilder 2005), persimmon (Jabbari Firoozjah and Mohammadi 2015) and forest trees (Kazemzadeh Chakusari 2017). This study represents the first record of *E. vitis* on walnut trees worldwide.

Results of our study showed that fruiting bodies of Botryosphaeriaceae species were quite frequent on wood debris. This result is consistent with reported results in studies conducted on forest, fruit and grapevine debris that emphasized the fact the fungi belonging to the Botryosphaeriaceae family survive well on wood debris (Santini et al. 2008). For example, *Diplodia sapinea* was isolated from pruning debris of *Prunus* spp. (Damm et al. 2007), *N. parvum* from flooded gum debris (Perez et al. 2008), *N. parvum* and *Sphaeropsis porosa* from grapevine pruning debris (Van Niekerk et al.

2003, 2004). Hence, wood debris can play important role facilitating overwintering of Botryosphaeriaceae in nut crops orchards, thus being a primary inoculum source to initiate infections.

Preliminary pathogenicity results showed that, all tested isolates caused wood discoloration on detached shoots of almond, pistachio and walnut. But, also revealed significant differences in the degree of virulence among species and among isolates of the same species, which is consistent with results obtained for other fungal trunk pathogens such as Botryosphaeriaceae (Úrbez-Torres and Gubler 2009) and Diatrypaceae species on grapevine (Trouillas and Gubler 2010b; Pitt et al. 2013), and Botryosphaeriaceae and *Phaeoacremonium* species on forest trees (Kazemzadeh Chakusari et al. 2017, 2019). On almond, *D. gallae*, *L. theobromae* and *N. parvum* were the most aggressive species, while *Eutypella* sp. 1 and *D. whitmanensis* did not produce significantly longer lesions than the controls. The high degree of aggressiveness observed in this study for *L. theobromae* is consistent with the findings reported by Rodriguez-Galvez et al. (2016) on mango stems in Peru, grapevine in California (Urbez-Torres and Gubler 2009) and peach trees in USA and China (Britton and Hendrix 1989; Wang et al. 2011). *Neofusicoccum parvum* and *N. mediterraneum* have been reported the most virulent species on almond trees in Spain (Olmo et al. 2016). Previous studies also showed that *N. luteum* and *N. parvum* are highly virulent on avocado (McDonald et al. 2009) and citrus (Adesemoye et al. 2014) in California, and on grapevine in California (Urbez-Torres and Gubler 2009), New Zealand (Billones et al. 2014) and Spain (Luque et al. 2009) and pistachio in Iran (Mohammadi et al. 2015).

Results of the pathogenicity tests on pistachio shoots showed that all inoculated species tested were pathogenic on this host. The lesions caused by *Eutypella* sp. 2 and one isolate of *B. dothidea* were longer than those caused by the other isolates. Results of previous studies showed that *B. dothidea* is one of the main pathogens of pistachio (Michailides 1991) but was reported to be weakly pathogenic to healthy vines, *Eucalyptus* and *Syzygium* in South Africa (Van Niekerk et al. 2004; Pavlic et al. 2007; Slippers et al. 2007).

Lasiodiplodia theobromae was the most virulent fungal species and caused the largest lesions on walnut shoots. Our results are consistent with previous pathogenicity studies, in which this species was shown to be one of the most virulent Botryosphaeriaceae species on grapevines (Leavitt 1990; Van Niekerk et al. 2004). According to pathogenicity trials conducted in California, *L. theobromae* has been reported as an aggressive pathogen on grapevines (Úrbez-Torres et al. 2009). This species is an important wood pathogen, which has been reported from more than 500 plant hosts, including nut trees (Punithalingam 1980, Phillips et al. 2013). The high virulence of *L. theobromae* indicates that this fungus should be considered one of the primary walnut trunk pathogens in Iran.

In summary, the current work represents the first detailed study concerning the isolation, identification and pathogenicity of fungal pathogens associated with branch cankers and trunk diseases on almond, pistachio and walnut, in Iran. Large number of Botryosphaeriaceae, Diatrypaceae and *Phaeoacremonium*, species were isolated from these hosts, and also more occasionally *C. hispanica* and *P. richardsiae*. New reports of some species in Iran or in new hosts improve

the current knowledge about the geographical distribution and host range of these species. Preliminary pathogenicity tests demonstrated that most of these fungi were pathogenic to almond, pistachio and walnut shoots. Therefore, more importance should be given to these pathogens in Iran, and specific management strategies should be included within the nut crops IPM management programs, with the aim of improving their sustainability.

Ethical statements

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Conflict of Interest: The authors declare that they have no conflict of interest.

Informed consent: Informed consent was obtained from all individual participants included in the study.

Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors.

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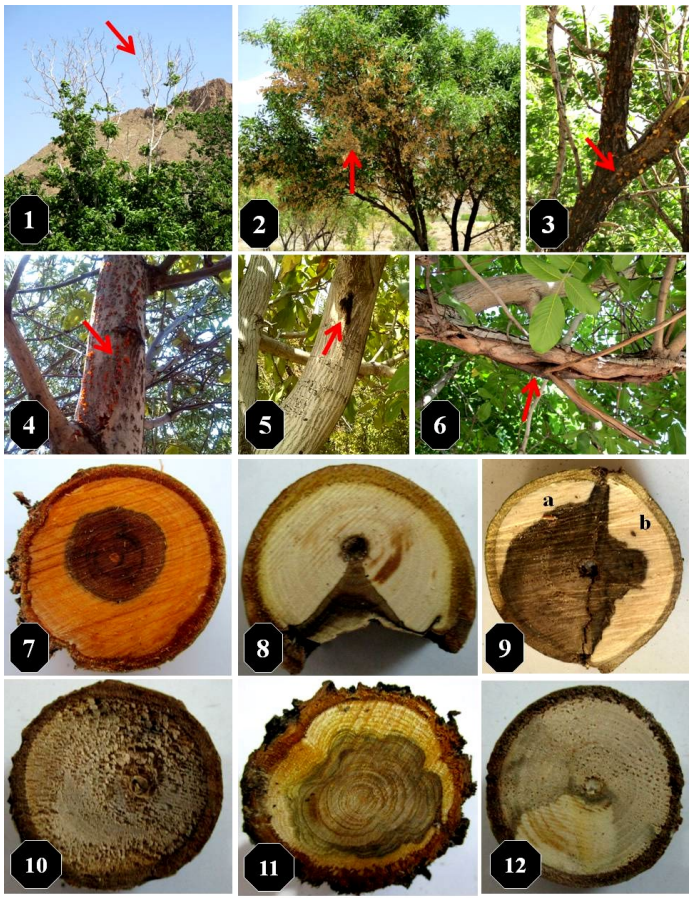

Fig. 1 Main branch canker and trunk disease symptoms found on nut trees (almond, pistachio and walnut). (1-6) external disease symptoms, 1) branch dieback on walnut, 2) yellowing on almond, 3-5) gumming, 3 on almond, 4 and 5 on walnut, 6) branch canker on walnut, (7-12) internal disease symptoms, 7) central necrosis on almond, 8) v-shaped necrosis on walnut, 9) co-occurrence of irregular wood necrosis (a) and black spot (b) on walnut, 10) wood decay on walnut, 11) brown to black wood streaking on almond, 12) watery necrosis on walnut.

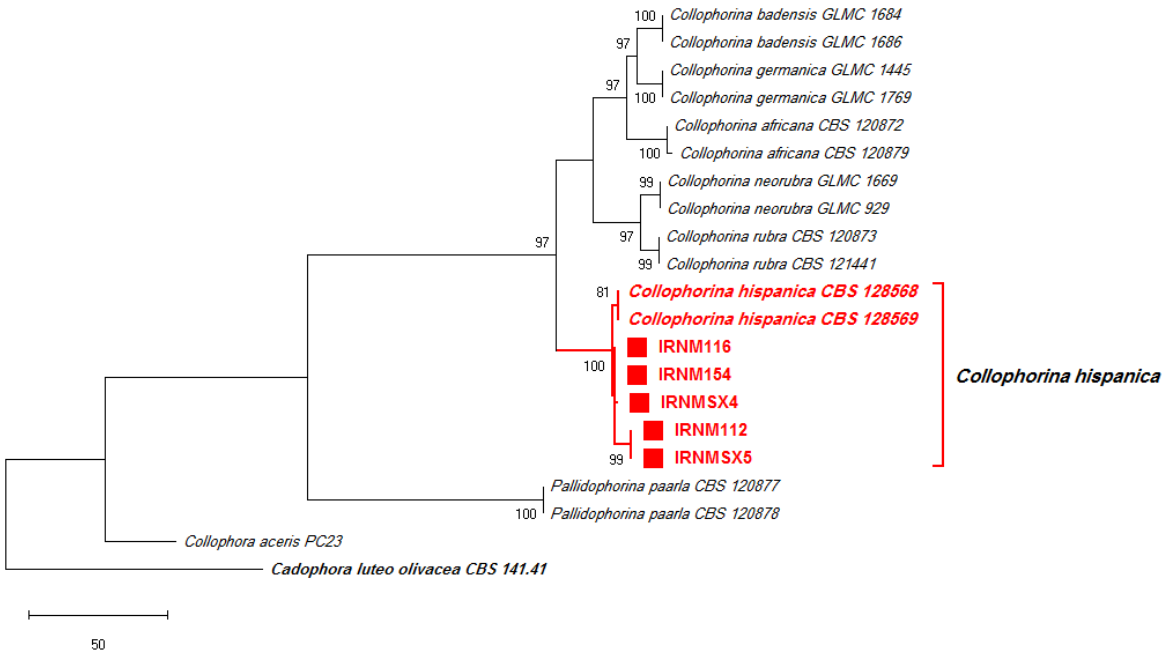

Fig. 2 One of the most parsimonious trees for *Collophorina* obtained from combined ITS and *tef* sequence data. Bootstrap support (1000 replicates) above 70 % are shown at the nodes. *Cadophora luteo olivacea* (CBS141.41) was used as outgroup and Iranian isolates obtained in this study shown in red color. Bar represents 50 changes.

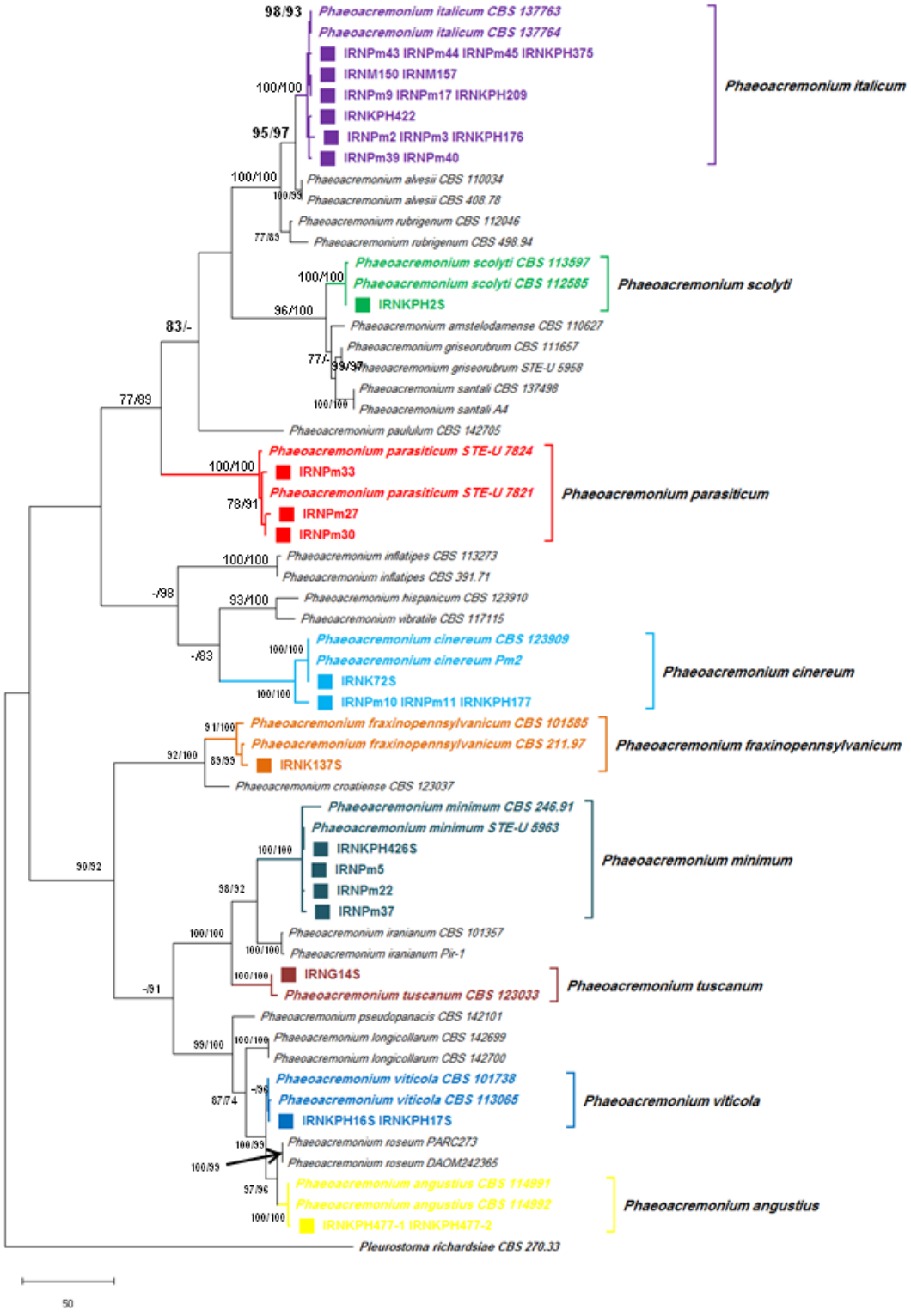

Fig. 3 One of the most parsimonious trees for *Phaeoacremonium* obtained from combined BT and ACT sequence data. ML/MP bootstrap support (1000 replicates) above 70 % are shown at the nodes. *Pleurostoma richardsiae* (CBS 270.33) was used as outgroup and Iranian isolates obtained in this study shown in color. Bar represents 50 changes.

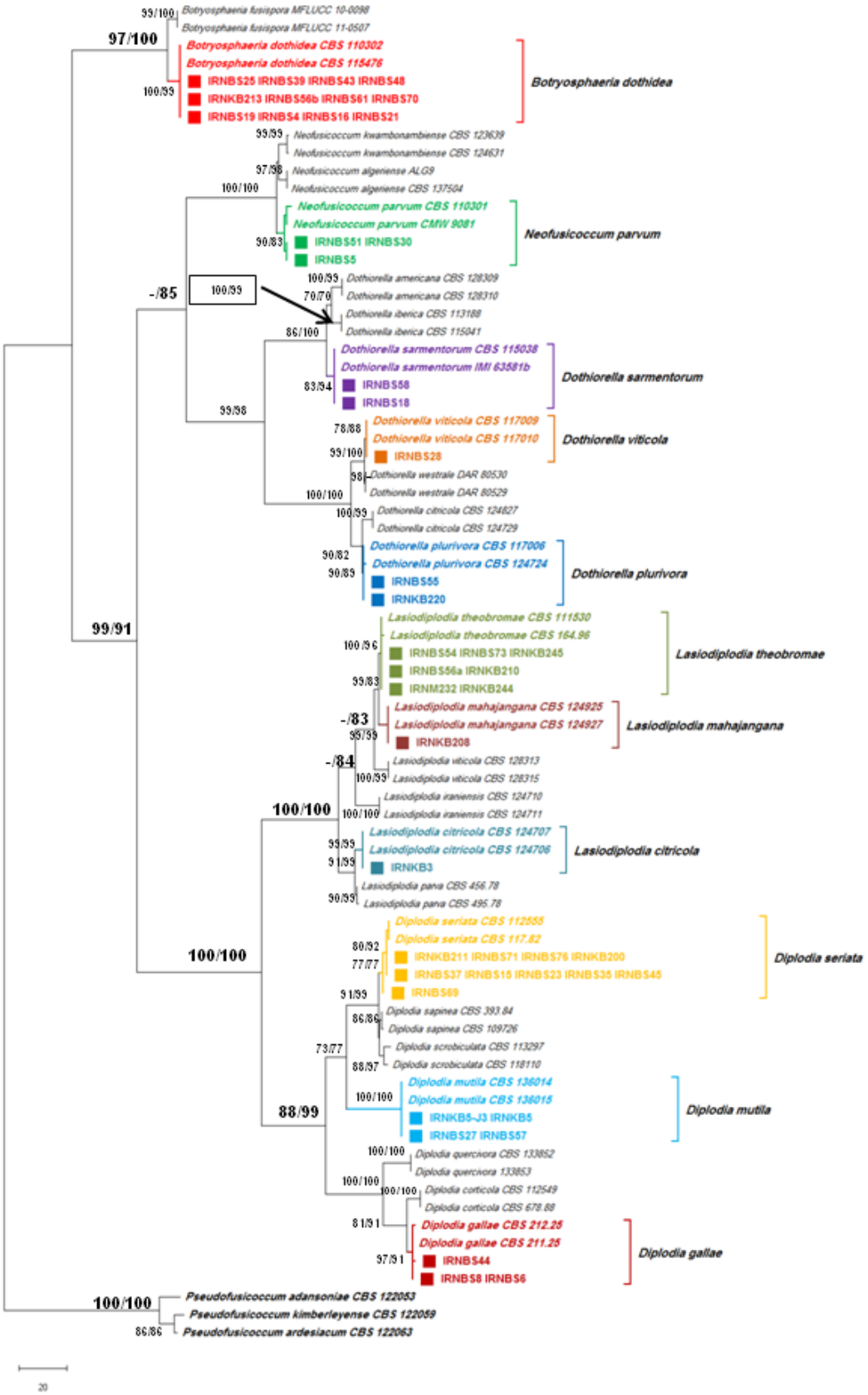

Fig. 4 One of the most parsimonious trees for Botryosphaeriaceae obtained from combined ITS and *tef* sequence data. ML/MP bootstrap support (1000 replicates) above 70 % are shown at the nodes. *Pseudofusicoccum adansoniae* (CBS 22055), *P. ardesiacum* (CBS 122062) and *P. kimberleyense* (CBS 122058**)** was used as outgroups and Iranian isolates obtained in this study shown in color. Bar represents 20 changes

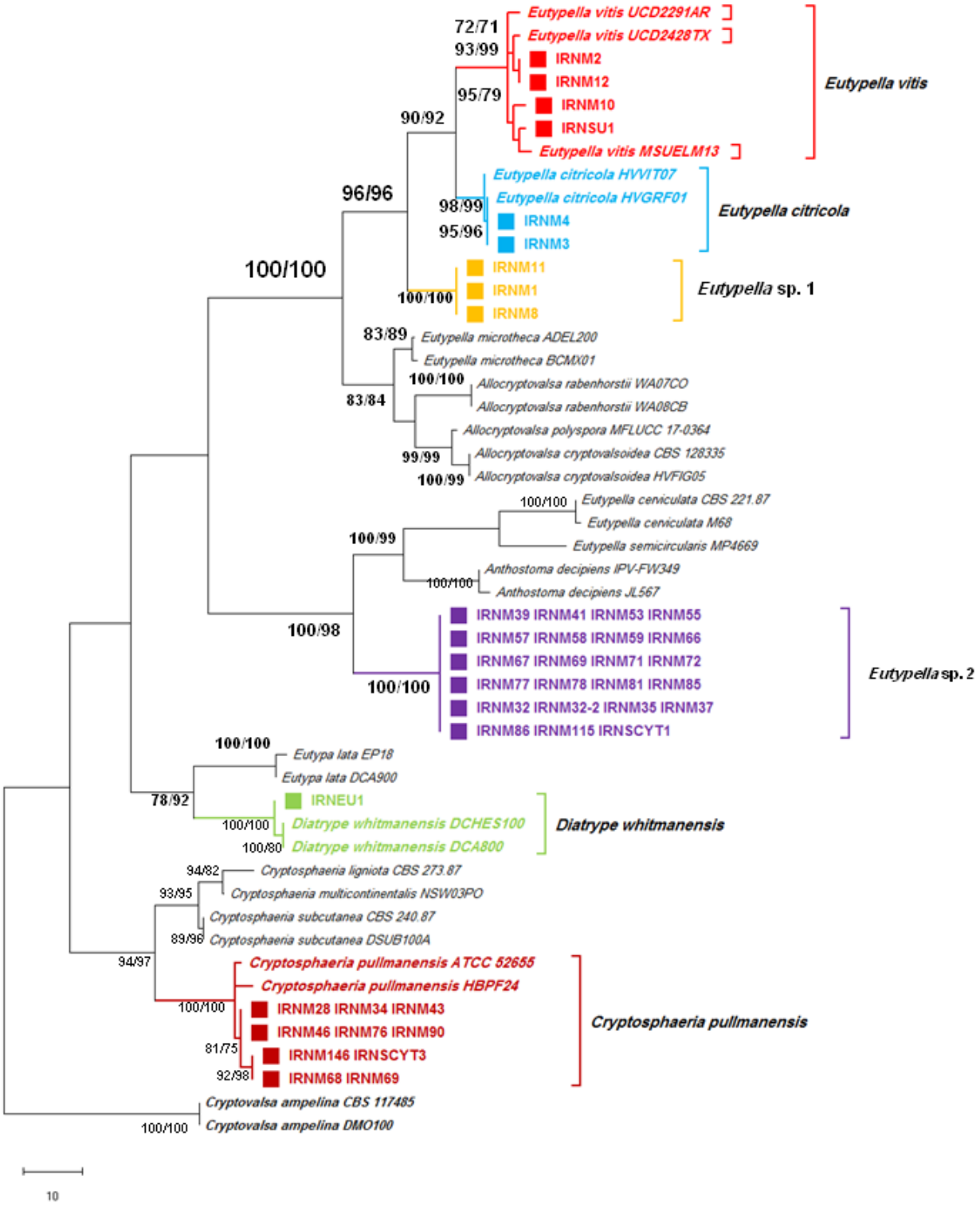

Fig. 5 One of the most parsimonious trees for Diatrypaceae obtained from ITS sequence data. ML/MP bootstrap support (1000 replicates) above 70 % are shown at the nodes. *Cryptovalsa ampelina* (CBS 117485, DMO100) was used as outgroups and Iranian isolates obtained in this study shown in color. Bar represents 10 changes

Fig. 6 Lesions obtained on pathogenicity tests of fungal species inoculated onto detached shoots of nut crops (almond, pistachio and walnut) 40 days after inoculation: (1-16) almond, 1) control (the arrow shows the point of inoculation) compared to wood discoloration caused by 2) *Botryosphaeria dothidea*, 3) *Collophorina hispanica* (IRNM112), 4) *Diatrype whitmanensis*, 5) *Diplodia gallae*, 6) *Diplodia mutila*, 7) *Diplodia seriata* (IRNBS15), 8) *Eutypella* sp. group1, 9) *Lasiodiplodia theobromae*, 10) *Neofusicoccum parvum*, 11) *Phaeoacremonium angustius*, 12) *Phaeoacremonium italicum*, 13) *Phaeoacremonium minimum*, 14) *Phaeoacremonium parasiticum*, 15) *Phaeoacremonium viticola*, 16) *Pleurostoma richardsiae* (IRNKPH222), (17-24) pistachio, 17) control, 18) *Botryosphaeria dothidea* (IRNBS61), 19) *Diplodia seriata*, 20) *Eutypella* sp. group 2, 21) *Eutypella citricola*, 22) *Phaeoacremonium minimum*, 23) *Phaeoacremonium parasiticum*, 24) *Phaeoacremonium viticola*, (25-48) walnut, 25)control, 26) *Botryosphaeria dothidea* (IRNBS19), 27) *Cryptosphaeria pullmanensis*, 28) *Diplodia gallae*, 29) *Diplodia seriata* (IRNBS23), 30) *Dothiorella plurivora* (IRNBS55), 31) *Dothiorella sarmentorum*, 32) *Dothiorella viticola*, 33) *Eutypella* sp. group1, 34) *Eutypella* sp. group 2, 35) *Eutypella vitis*, 36) *Lasiodiplodia citricola*, 37) *Lasiodiplodia mahajangana*, 38) *Lasiodiplodia theobromae*, 39) *Neofusicoccum parvum*, 40) *Phaeoacremonium cinereum* (IRNPm10), 41) *Phaeoacremonium fraxinopennsylvanicum*, 42) *Phaeoacremonium italicum* (IRNKPH176), 43) *Phaeoacremonium minimum*, 44) *Phaeoacremonium parasiticum*, 45) *Phaeoacremonium scolyti*, 46) *Phaeoacremonium tuscanum*, 47) *Phaeoacremonium viticola*, 48) *Pleurostoma richardsiae* (IRNPm42).

Table 1 Mean lesion length and re-isolation frequencies of fungal species inoculated onto almond tree shoots in the pathogenicity test.

Isolates		Mean lesion length (mm)			Re-isolation frequency (%)
Species	**Code (IRN)**	**Up**	**Down**	**Total**	
Botryosphaeria dothidea	BS4*	49.60 **BCDEF**	63.00 **B**	112.60 **BCD**	71.0
Collophorina hispanica	M112	34.60 **DEFG**	29.00 **CDE**	64.00 **DEF**	57.0
	MSX4	62.40 **ABC**	35.60 **BCD**	98.00 **BCDE**	64.0
Diatrype whitmanensis	EU1	17.60 **GH**	15.80 **DE**	33.40 **FG**	57.0
Diplodia gallae	BS8*	78.80 **A**	126.20 **A**	205.00 **A**	57.0
Diplodia mutila	KB5	36.60 **CDEFG**	63.80 **B**	100.40 **BCDE**	57.0
Diplodia seriata	BS15	52.40 **ABCDEF**	50.40 **BC**	102.80 **BCD**	43.0
	BS35*	36.00 **CDEFG**	43.40 **BCD**	79.40 **BCDEF**	57.0
Eutypella sp. 1	M32-2	35.20 **DEFG**	16.80 **DE**	52.00 **EFG**	64.0
Lasiodiplodia theobromae	BS54	62.40 **ABC**	116.80 **A**	179.20 **A**	71.0
Neofusicoccum parvum	BS30*	67.20 **AB**	102.80 **A**	170.00 **A**	71.0
Phaeoacremonium angustius	KPH477-1	32.60 **EFG**	31.80 **BCDE**	64.40 **CDEF**	71.0
Phaeoacremonium italicum	Pm39	28.40 **FGH**	42.00 **BCD**	70.40 **BCDEF**	64.0
Phaeoacremonium minimum	KPH178	40.20 **BCDEFG**	31.40 **BCDE**	71.60 **BCDEF**	86.0
Phaeoacremonium parasiticum	Pm33	61.00 **ABCD**	55.00 **BC**	116.00 **B**	64.0
Phaeoacremonium viticola	KPH17S	41.00 **BCDEFG**	39.20 **BCD**	80.20 **BCDEF**	71.0
Pleurostoma richardsiae	KPH222	45.80 **BCDEF**	63.00 **B**	108.80 **BCD**	57.0
	Pm24	55.80 **ABCDE**	57.20 **BC**	113.00 **BC**	64.0
Control	-	1.80 **H**	2.40 **E**	4.20 **G**	0
LSD (P<0.05)	-	27.104	32.991	48.857	

Different letters in bold face indicate significant differences only within a column at $P = 0.05$.

* Isolates obtained from wood debris.

Table 2 Mean lesion length and re-isolation frequencies of fungal species inoculated onto pistachio tree shoots in the pathogenicity test

Isolates		Mean lesion length (mm)			Re-isolation frequency (%)
Species	**Code (IRN)**	**Up**	**Down**	**Total**	
Botryosphaeria dothidea	BS48	37.600 **AB**	25.200 **BC**	62.80 **BCD**	57.0
	BS61*	41.800 **AB**	54.200 **A**	96.00 **A**	50.0
Diplodia seriata	BS71*	31.800 **AB**	28.800 **BC**	60.60 **BCD**	43.0
Eutypella sp. 2	M1	45.800 **A**	62.600 **A**	108.40 **A**	78.0
Eutypella citricola	M4	14.800 **D**	19.600 **C**	34.40 **D**	43.0
Phaeoacremonium minimum	Pm37	32.200 **AB**	32.000 **BC**	64.20 **BC**	64.0
Phaeoacremonium parasiticum	Pm30	16.000 **CD**	21.600 **BC**	37.60 **CD**	71.0
Phaeoacremonium viticola	Pm26	29.800 **BC**	36.600 **B**	66.40 **B**	50.0
Control	-	2.000 **D**	1.600 **D**	3.60 **E**	0
LSD (P<0.05)	-	14.891	16.615	28.511	

Different letters in bold face indicate significant differences only within a column at $P = 0.05$.
* Isolates obtained from wood debris.

Table 3 Mean lesion length and re-isolation frequencies of fungal species inoculated onto walnut tree shoots in the pathogenicity test.

Isolates		Mean lesion length (mm)			Re-isolation frequency (%)
Species	**Code (IRN)**	**Up**	**Down**	**Total**	
Botryosphaeria dothidea	KB213	16.20 **NOP**	32.80 **IJKLM**	49.00 **MN**	75.0
	BS19*	87.20 **ABC**	115.80 **BC**	203.00 **ABC**	78.0
Cryptosphaeria pullmanensis	SCYT3	75.80 **ABCDE**	85.60 **CDEF**	162.40 **CDEF**	66.6
Diplodia gallae	BS6	95.60 **A**	127.00 **AB**	222.60 **AB**	78.0
Diplodia seriata	KB211	23.80 **MNOP**	29.80 **JKLM**	53.60 **MN**	58.0
	BS23*	64.20 **DEFG**	90.40 **CDEF**	154.60 **CDEFG**	64.0
Dothiorella plurivora	KB220	20.20 **MNOP**	25.80 **LM**	46.00 **MN**	78.3
	BS55*	56.60 **EFGHIJ**	59.20 **FGHIJK**	115.80 **FGHIJK**	64.0
Dothiorella sarmentorum	BS18*	58.40 **DEFGHI**	78.40 **DEFGH**	136.80 **EFGH**	57.0
Dothiorella viticola	BS28	30.60 **LMNO**	34.40 **IJKLM**	64.60 **LM**	50.0
Eutypella sp. 1	M32-1	39.40 **HIJKLM**	35.00 **IJKLM**	74.40 **JKLM**	50.0
Eutypella sp. 2	M8*	89.20 **AB**	106.00 **BCD**	195.20 **ABCD**	50.0
Eutypella vitis	SU1	72.60 **BCDEF**	75.00 **DEFGH**	147.60 **DEFG**	55.0
Lasiodiplodia citricola	KB3	36.00 **JKLMN**	31.60 **IJKLM**	67.60 **KLM**	75.0
Lasiodiplodia mahajangana	KB208	58.00 **EFGHIJ**	51.80 **GHIJKL**	109.80 **GHIJKL**	71.3
Lasiodiplodia theobromae	KB245	88.00 **ABC**	156.40 **A**	244.40 **A**	93.0
Neofusicoccum parvum	BS5	80.40 **ABCD**	95.00 **BCDE**	175.40 **BCDE**	86.0
Phaeoacremonium cinereum	K72S	66.20 **CDEF**	84.40 **CDEFG**	150.60 **DEFG**	50.0
	KPH177	13.20 **OP**	29.40 **JKLM**	42.60 **MN**	58.3
	Pm10	59.60 **DEFGH**	75.60 **DEFGH**	135.20 **EFGHI**	57.0
Phaeoacremonium fraxinopennsylvanicum	K137S	37.80 **HIJKLMN**	47.20 **HIJKL**	85.00 **IJKLM**	57.0

Table 3 Continued.

Isolates		Mean lesion length (mm)			Re-isolation frequency (%)
Species	**Code (IRN)**	**UP**	**Down**	**Total**	
Phaeoacremonium italicum	KPH176	33.40 **KLMNO**	26.60 **KLM**	60.00 **LMN**	58.3
	KPH209	13.60 **OP**	30.00 **JKLM**	43.60 **MN**	75.0
Phaeoacremonium minimum	KPH426S	63.60 **DEFG**	61.00 **FGHIJ**	124.60 **EFGHIJ**	64.0
Phaeoacremonium parasiticum	Pm27	58.80 **DEFGHI**	64.60 **EFGHI**	123.40 **FGHIJ**	71.0
Phaeoacremonium scolyti	KPH2S	42.20 **GHIJKLM**	49.80 **HIJKL**	92.00 **HIJKLM**	78.0
Phaeoacremonium tuscanum	G14S	53.20 **FGHIJK**	70.60 **EFGH**	123.80 **FGHIJ**	86.0
Phaeoacremonium viticola	KPH16S	37.40 **IJKLMN**	34.00 **IJKLM**	71.40 **KLM**	58.3
Pleurostoma richardsiae	KPH423	41.20 **HIJKLM**	21.40 **LM**	62.60 **LM**	66.6
	Pm42	51.40 **FGHIJKL**	79.20 **DEFGH**	130.60 **EFGHI**	64.0
Control	-	5.20 **P**	6.20 **M**	11.40 **N**	0
LSD (P<0.05)	-	22.033	33.386	50.916	

Different letters in bold face indicate significant differences only within a column at $P = 0.05$.

* Isolates obtained from wood debris.