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First report of *Graphium carbonarium* associated with walnut dieback in Iran

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Abstract: Walnut tree is one of the most important nut crops in Iran. Dieback and decline of walnut trees are some of the factors limiting the cultivation and sustainability of this crop. During 2017 and 2018, field surveys were conducted on walnut orchards in Yazd province to study of fungal pathogens associated with diseased trees. Wood samples were collected from diseased branches showing canker, dieback and gumming symptoms. In the laboratory, affected branches were cut transversally and infected wood tissues were cut into small pieces. Wood pieces were plated on a potato-dextrose-agar (PDA) after surface sterilization. In this study, 10 isolates of a fungus were obtained from affected trees. Based on morphological and molecular (for two selected Iranian isolates based on ITS-rDNA and *tef1-α* gene sequences) characteristics, all isolates were identified as *Graphium carbonarium*. Based on literature reviews, this is the first report of this species associated with necrotic wood of walnut trees in the world.

Keywords: Canker, *Graphium*, gumming, ITS-rDNA, *tef1-α*.

INTRODUCTION

Walnut (*Juglans regia*, Juglandaceae), as one of the most important nut crops, has long been of interest to humans to produce wood and fruit. According to FAO (2018), Iran, with 150,000 ha and 405,000 tons of walnut, is the third-largest producer of walnut in the world.

The genus *Graphium sensu lato* has been identified by usually well-developed dark synnemata, producing single-celled conidia in slimy masses. *Graphium* species have been isolated from soil, plant debris, woody substrate and galleries of bark beetles (Jacobs et al. 2003). *Graphium carbonarium* Paciura, Z.W. de Beer, X.D. Zhou & M.J. Wingf. was first identified associated with a *Pissodes* sp. on *Salix babylonica* (Paciura et al. 2010). This species has also been reported from *Tsuga dumosa* (Paciura et al. 2010), *Larix olgensis* (Liu et al. 2016) from China, *Ricinus communis* in China and *Acacia auriculiformis* trees in Vietnam (Lynch et al. 2016). In the current study, 10 isolates of a *Graphium* species were isolated from walnut trees showing decline symptoms in Yazd province. The objective of this study was to identify these isolates using morphological and molecular characteristics.

MATERIALS AND METHODS

Sampling and fungal isolation

During 2017 and 2018, field surveys were conducted on walnut orchards in Yazd province to study of fungal pathogens associated with trees showing decline symptoms. Samples were collected from branches of trees with canker, dieback and gumming symptoms. Fungal isolations were conducted from internal wood necrotic tissues. In the laboratory, small wood segments (5×5 mm) were cut from affected tissues and surface-sterilized in 0.5 % sodium hypochlorite for 2 min followed by two rinses in sterile distilled water and then placed on potato dextrose agar (PDA, Merck, Germany) amended with streptomycin sulphate. The cultures were incubated in the dark at 25°C. For further study, pure cultures were obtained from each isolate based on a single spore method.

Morphological and molecular identification and phylogeny

The putative identities of isolates were based on morphology following methods of Paciura et al. (2010). In order to molecular identification of the isolates, the total genomic DNA was first extracted from the aerial mycelium using a CTAB method (Doyle & Doyle 1990). All DNA samples were incubated at −17°C until used for PCR amplification.

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The internal transcribed spacer 1 and 2, including the intervening 5.8S nrDNA gene (ITS-rDNA) and a partial sequence of the translation elongation factor 1-alpha (*tef-1α*) gene, were amplified using primer sets ITS1/ITS4 (White et al. 1990) and EF1-728F/EF1-986R (Carbone & Kohn 1999), respectively. PCR products were purified and sequenced by 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.

For phylogenetic analyses, individual loci sequences obtained in this study and those references retrieved from GenBank were aligned using default settings of Clustal W algorithm (Thompson et al. 1994) included within MEGAX software package (Kumar et al. 2018). The alignments were manually checked and improved where necessary. Phylogenetic analyses for each locus and concatenated datasets were based on Maximum Likelihood (ML) and Maximum Parsimony (MP). 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). All sequences were deposited in GenBank (Table 1).

RESULTS AND DISSCUSSION

Field surveys were conducted on walnut orchards in Yazd province. The most important external disease symptoms observed on walnut trees were gumming, branch cankers and dieback. Internal symptoms included central, irregular, watery and v-shaped necrosis, which were observed in cross-sections of diseased branches (Fig. 1).

Graphium isolates produced dark gray-olive colonies with a white margin, aerial mycelium and abundant synnemata on PDA. Conidia aseptate, hyaline, curved cylindrical, aggregate in a hyaline mucilaginous mass at the apices of the synnemata. Based on morphological and cultural features, the fungal isolates identified tentatively as *Graphium* sp. (Paciura et al. 2010). Iranian isolates had synnematous anamorph in culture and sexual structures were never observed. Conidiophores organized in synnemata, that generally formed in groups and sometimes singly. Synnemata had 125-290 µm long and 43–59 µm wide at the apex. Conidia aseptate, hyaline, curved, cylindrical with truncated bases, 3.5-6 µm × 1-3.5 µm (Fig. 2). The optimum growth temperature is 25–30°C. Colonies reaching a radius of 4.5-6 mm in 7 d and 9.5-10.5 mm in 14 d at 25 °C. *Graphium carbonarium* is most closely related to *G. basitruncatum* (Matsush.) Seifert & G. Okada and *G. euwallaceae* Twizeyim., S.C. Lynch & Eskalen. However, they also have morphological differences. *Graphium carbonarium* has larger synnemata and conidia than *G. basitruncatum*. The latter species is characterized by conidiophores (70-)72–131(-158) µm in length, conidiogenous apparatus (19-)24–45(-56) µm wide and conidia of 5–6×1–2 µm in size (Paciura et al. 2010). Conidia in *G. euwallaceae* are also shorter and slender compared to *G. basitruncatum* and *G. carbonarium* (Lynch et al. 2016).

DNA sequence comparisons were conducted to confirm the identity of these isolates. The two individual phylogenetic analyses (ITS and *tef-1α*) resulted in a similar tree topology (data not shown). Sequences of two Iranian isolates, 17 reference isolates of *Graphium* spp. (include of nine species) and *Pseudallescheria boydii* (Shear) McGinnis, A.A. Padhye & Ajello (as outgroup) were aligned. The combined alignment consisted of 1082 characters, including gaps (ITS: 563 and *tef-1α*: 519). Of these, 626 were constant and 276 parsimony informative.

Table 1. Origins, host and GenBank accession numbers of the *Graphium* strains used in phylogenetic analyses (Iranian isolates are shown in bold type).

Isolates		Host	Origion	Genbank Accession Number	
Species	Code			ITS	*tef1-α*
Graphium adansoniae	CMW30618[T]	*Adasonia digitata*	South Africa	KM592371	KM592363
	CMW30620	*A. digitata*	South Africa	GQ200613	HM630597
Graphium basitruncatum	JCM9300	Forest soil	Solomon Islands	AB038427	KJ131248
Graphium carbonarium	CMW12420[T]	*Salix babylonica / Pissodes* sp.	China	FJ434979	HM630603
	CMW12418	*S. babylonica / Pissodes* sp.	China	FJ434980	HM630602
	IRNPm47	***Juglans regia***	**Iran**	**MT605368**	**MT625161**
	IRNPm48	***J. regia***	**Iran**	**MT605369**	**MT625162**
Graphium euwallaceae	UCR 2980 [T]	*Acasia* sp.	Vietnam	KM592371	KM592363
	UCRFD97	*Acasia floribunda*	California	KF540225	KF534806
Graphium fimbriisporum	CMW5605[T]	*Picea abies*	France	AY148177	HM630590
	CMW5606	*P. abies*	Austria	AY148180	HM630591
Graphium larics	CMW5601[T]	*Larix deddua*	Austria	AY148162	HM630588
	CMW5603	*L. deddua*	Austria	AY148182	HM630589
Graphium madagascariense	CMW30628[T]	*Adasonia rubrostipa*	Austria	HM630606	HM630595
	CMW30629	*A. rubrostipa*	Austria	HM630607	HM630594
Graphium penicillioides	CMW5292[T]	*Populus nigra*	Czech Republic	HQ335310	HM630600
	CMW5295	*P. nigra*	Czech Republic	HQ335311	HM630601
Graphium pseudormiticum	CMW503[T]	*Pinus* sp.	South Africa	AY148186	HM630586
Pseudallescheria boydii	CBS 101.22	*Homo sapiens*	USA	AM887718	EF151369

[T] Ex-type strains

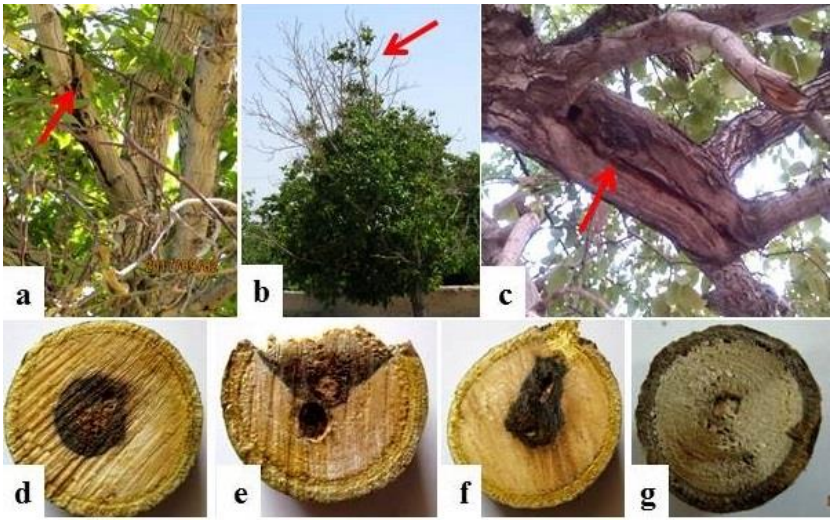

Fig. 1. Main branch canker and trunk disease symptoms found on walnut trees. a-c. external disease symptoms; a. gumming; b. branch dieback; c. branch canker; d-g. internal wood lesion types; d. central necrosis; e. v-shaped necrosis; f. irregular wood necrosis; g. watery necrosis.

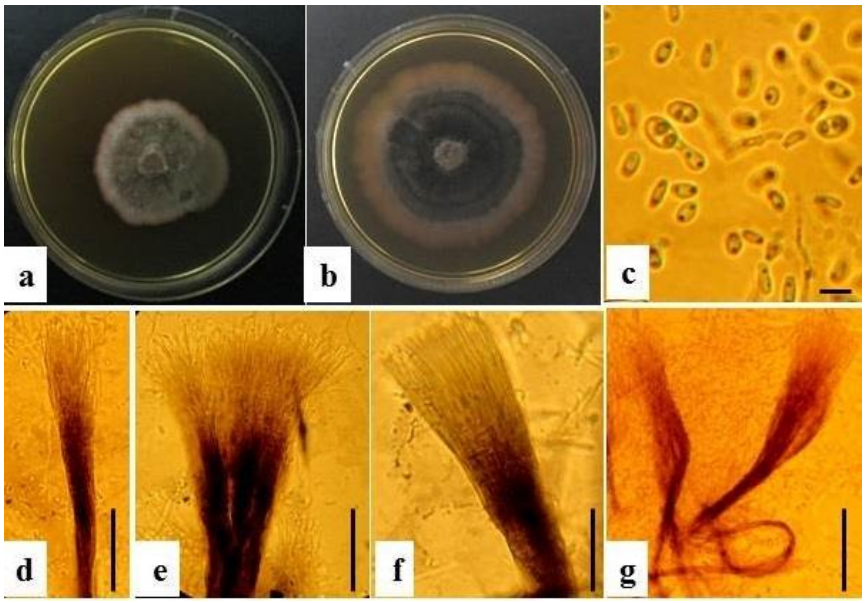

Fig. 2. *Graphium carbonarium.* Colony on PDA after a. 14 days and b. 28 days, c. Conidia, d-g. Synnemata. — Scale bar = 40µm, c = 5µm.

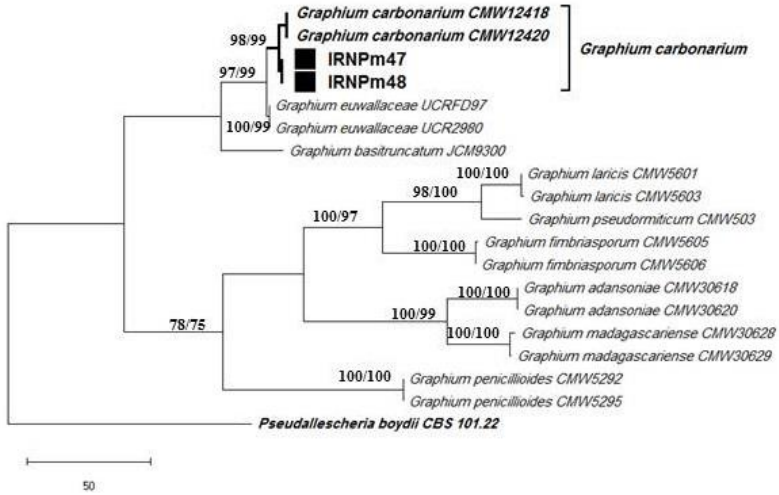

Fig. 3. One of the most parsimonious trees for *Graphium* obtained from combined ITS-rDNA and *tef1-α* sequence data. ML/MP bootstrap support (1000 replicates) above 70 % are shown at the nodes. *Pseudallescheria boydii* (CBS 101.22) was used as outgroup and Iranian isolates (IRNPm47 and IRNPm48) obtained in this study and isolates of *G. carbonarium* retrieved from GenBank shown in bold type. Bar represents 50 changes.

Maximum parsimony analysis resulted in three equally most parsimonious trees (TL=645, CI=0.817; RI=0.912, RC=0.745). MP tree of the respective datasets is presented as Fig. 3, with bootstrap results from the ML and MP trees. Based on MP analyses, the Iranian isolates clustered with the reference isolates of *Graphium carbonarium*. In this study, *G. carbonarium* collected from walnut trees in Yazd

province. This species was first identified and described by Paciura et al. (2010) associated with a *Pissodes* sp. on *Salix babylonica*. Phylogenetic analyses reveal that *G. carbonarium* is distinct but closely related to *Graphium euwallaceae* and *Graphium basitruncatum*. This species has also been reported from *Tsuga dumosa* (Paciura et al. 2010) and *Larix olgensis* (Liu et al. 2016) from China. In 2016, *G. carbonarium* was isolated from *Ricinus communis* in China and *Acacia auriculiformis* trees in Vietnam (Lynch et al. 2016). *Graphium basitruncatum* was first described from forest soil in the Solomon Islands as *Stilbum basitruncatum* Matsush. (Matsushima 1971). This species has been isolated from a patient with leukemia in Canada, confirming that this species can act as an opportunistic human pathogen (Deepali et al. 2007). In a study conducted by Lynch et al. (2016), *G. euwallaceae* was reported as a pathogen of avocado and box elder (Lynch et al. 2016). Based on the literature review, the present study has shown that the walnut trees can also be considered as a new host for this species in the world.

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اولین گزارش از حضور گونه *Graphium carbonarium* همراه با سرخشکیدگی درختان گردو در ایران

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چکیده: درخت گردو یکی از مهمترین درختان میوه آجیلی در ایران می‌باشد. سرخشکیدگی و زوال درختان گردو به عنوان فاکتورهای محدودکننده کشت و پایداری این محصول شناخته می شوند. به منظور مطالعه عوامل بیماری‌زای قارچی همراه با درختان بیمار، بعضی از باغ‌های گردو در استان یزد در طول سال‌های ۱۳۹۶ و ۱۳۹۷ مورد بررسی قرار گرفتند. نمونه برداری از شاخه‌های درختان بیمار با نشانه‌های شانکر، سرخشکیدگی و صمغ‌زدگی انجام شد. شاخه‌های آلوده در آزمایشگاه به صورت عرضی برش داده شدند و بافت تغییر رنگ یافته چوب به قطعات کوچک بریده شدند. قطعات کوچک چوب بعد از سترون کردن سطحی بر روی محیط کشت سیب‌زمینی-دکستروز-آگار (PDA) قرار داده شدند. در این مطالعه، ۱۰ جدایه از یک گونه قارچی از درختان بیمار به دست آمد. بر اساس ویژگی‌های ریخت‌شناسی و ملکولی (برای دو جدایه ایرانی انتخاب شده بر اساس ناحیه ITS-rDNA و ژن *tef-1α*)، همه جدایه‌ها به عنوان گونه *Graphium carbonarium* شناسایی شدند. بر اساس بررسی منابع موجود، این مطالعه اولین گزارش از وجود این گونه همراه با نکروز بافت چوب درختان گردو در دنیا می باشد.

کلمات کلیدی: شانکر، *Graphium*، صمغ‌زدگی، ITS-rDNA، *tef-1α*.

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