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Morphological and molecular characterization of *Uzbekistanica* spp. isolated from walnut trees

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Abstract: During surveys from walnut orchards of Hamedan and Kermanshah provinces, black spots on twigs of walnut trees were observed between 2018 and 2019. Due to the prevalence of these symptoms on the twigs of walnut trees, the samples were transported to the mycology laboratory for further investigations. After isolation and purification on PDA medium, 19 isolates were obtained from symptomatic twigs. Microscopic observations were conducted for grouping and morphological identifications. Based on morphological traits, two representative isolates were selected for molecular identifications. Molecular identification based on Internal Transcribed Spacer (ITS) region, indicated that these isolates were *Uzbekistanica vitis-viniferae* and *U. yakutkhanika*. Pathogenicity examinations were showed these isolates were pathogenic on walnut shoots in the laboratory conditions. To the best of our knowledge, this is the first report of *Uzbekistanica* as a new genus for Funga of Iran. Moreover, walnut tree is new host for *U. vitis-viniferae* and *U. yakutkhanika*.

Key words: *Juglans regia*, *Melanommataceae*, *Pleosporales*, ITS, *Uzbekistanica*

INTRODUCTION

Persian walnut (*Juglans regia* L.), is an important and nutritionally valuable crop in the world as well as Iran. At present, China is considered as one of the main walnuts producing countries and Iran is the second-largest country in regard of walnut production (Ahmad shah et al. 2021). *Uzbekistanica* was introduced by Wanasinghe et al. (2018) with *U. rosae-hissaricae* as the type species. This genus is saprobic on terrestrial habitates (Wanasingh et al. 2018) and saprobic or weak pathogenic on dead twigs (Hyde et al. 2020). *Uzbekistanica* is a new genus belonging to *Melanommataceae*, which introduced as a new genus to accommodate species with broadly oblong ascomata, filamentous, pseudoparaphyses, cylindrical to cylindrical-clavate asci, muriform, ellipsoidal, yellow-brown to brown ascospores, and globose conidiomata with hyaline 1-septate, sepia or brown conidia (Wanasinghe et al. 2018). The name was chosen because it was first isolated from Uzbekistan. The *Melanommataceae* are a family of fungi in the order *Pleosporales* and are saprobic on wood and bark (Cannon & Kirk 2007). Four species of this genus have been reported until now. Two species were described from Uzbekistan, namely *U. yakutkhanika* and *U. rosae-hissaricae* (Wanasinghe et al. 2018). *U. vitis-viniferae* was from Ukraine and *U. pruni* was reported from Russia as the third and fourth species to this complex respectively (Crous et al. 2020, Hyde et al, 2020). During the sampling from walnut orchards, significant symptoms were observed on twigs of walnut trees in Iran. Due to the frequency of these symptoms, the main objective of this study was isolation and identification of the causative agent of these symptoms.

MATERIALS AND METHODS

Sample collection, isolation and morphological characterization

During repeated sampling from walnut orchards of Hamedan and Kermanshah provinces in Iran, common occurrence of black spot symptoms was observed on the twigs of walnut trees. Specimens were collected and transported to mycology laboratory for further investigations. To identify the causative agent of these symptoms, isolation was conducted on the basis of following method: before isolation, samples were washed in tap water to remove surface contaminations. Small fragments ($5mm^2$) were cut from the edge between healthy and symptomatic tissue, surface sterilized in 70% ethanol for 30 s, then in 1% sodium hypochlorite for 3 min and finally rinsed in sterile water 3 times (3 min each). The surface-disinfected tissues were dried on sterilized filter paper and incubated on potato dextrose agar (PDA; 200 g potatoes, 20 g dextrose, 15

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g agar, 1 l water) in petri dishes, four pieces in per dish and three dishes for each sample. The dishes with tissues were incubated at 25 °C and examined daily for fungal growth. The fungal colonies were individually transferred to new plates of PDA. The new fungal cultures were purified by single spore method (Ho & Ko 1997) and the pure cultures were stored in the laboratory of Bu-Ali Sina University of Iran and were also deposited in the Iranian Research Institute of Plant Protection Culture Collection. The purified isolates were incubated in fresh PDA at 23–25 °C in the dark for seven days. After seven days, morphology of the colonies including color and microscopic characters produced by each isolate was examined with a BX53 microscope (Olympus, Tokyo, Japan) equipped with Nomarski differential interference optics and fungal structures, length and width of 30 randomly selected conidia of each isolate were examined and registered. Obtained isolates were screened on the basis of morphological features and two isolates (SB425 and SB435) were selected as the representative isolates for molecular studies.

DNA extraction, PCR and sequencing

To confirm the morphological identifications, genomic DNA of representative isolates was extracted using the described method of Moller (1997). Polymerase reaction chain (PCR) was conducted by amplifying the Internal Transcribed Spacer (ITS) region as described by Crous et al (2020). Representative isolates were identified based on ITS sequence data using the primer pair ITS1 and ITS4, which distinguished them from other groups of fungi. The PCR was carried out in a TC-512 thermal cycler (Techne, Germany) in a total volume of 25 µl. The 25 µl reaction volume contained 10 ng of genomic DNA, 1 µM of each primer, 0.2 mM of dNTPs (CinnaGen, Iran), 2.5 µL 10X PCR buffer, 2.5 mM $MgCl_2$ and 1 U Taq DNA polymerase (CinnaGen, Iran). PCR condition was set as follow: initial denaturation step at 94°C for 5 min, followed by 35 cycles at 94°C for 1 min, 56°C for 1 min, and 72°C for 1 min, with a final extension at 72°C for 10 min. The amplified DNA fragments were purified and sequenced by Microsynth Company (The Swiss DNA Company, Bern, Switzerland).

Phylogenetic analysis

Generated sequences in this study, were edited manually and adjustments were made where necessary in the BioEdit v.7.0.5.2 (Hall 1999). The sequences were first analyzed by Blastn search (http://www.ncbi.nlm.nih.gov/Blast.cgi) to confirm their identities and comparison with the previously reported isolates available in the NCBI. Obtained sequences in this study were deposited in the GenBank nucleotide database and accession numbers have been recorded. All sequences were aligned by using the CLUSTAL-W program (Kumar et al. 2016). ITS sequences of ex-type isolates as *Uzbekistanica* species obtained from GenBank were included in the analysis in addition to the two representative isolates of *Uzbekistanica* sequenced in this study.

Phylogenetic tree was produced with maximum likelihood (ML) analyses of sequence dataset in MEGA 7 (Kumar et al. 2016) with the Kimura-2-parameter nucleotide substitution model (Kimura 1980) and 1000 bootstrap replicates were performed to assess the statistical support for tree topology (Felsenstein 1985). *Monoseptella rosae* Wanas. Gafforov & K.D. Hyde (MFLUCC 0815-17) was chosen as outgroup.

Pathogenicity tests

All of the obtained isolates were examined for the investigation of pathogenicity. The pathogenicity assay of 7-day-old walnut isolates was conducted according to the previously described procedure (Bagherabadi et al. 2017). Fifty seven healthy excised shoots (each isolate in triplicates) of walnut trees were inoculated with obtained isolates in this study. For artificial inoculation, the surface of healthy shoots was disinfected with 70% ethanol and a wound in the shoots was produced with a sterile metal cork borer (5 mm diameter). Five-mm mycelium PDA plug from a 7-day-old culture of fungal isolates was placed into the fresh wound and three additional shoots were wounded and inoculated with a sterile 2% PDA plug and served as negative controls. The wound was wrapped with parafilm. Inoculated shoots were placed in a plastic container comprising moistened filter papers to keep the relative humidity high. The shoots in the containers were kept in laboratory conditions at 23–25 °C with natural daylight. To fulfill Koch's postulates, re-isolation was performed based on the same method as described for preliminary isolation. The experiment was repeated twice using the same methodology.

RESULTS

Disease symptoms, distribution and morphological studies

Black spots symptoms were observed on twigs of walnut trees distributed in Hamedan and Kermanshah provinces of Iran (Fig. 1). In total, 19 isolates were obtained from symptomatic twigs of walnut trees. Based on morphological characterizations, these isolates were grouped into two categories and morphological features confirmed that these isolates were similar to *Uzbekistanica* genus (Wanasinghe et al, 2018, Crous et al, 2020, Hyde et al, 2020). Based on current literature about this genus, these isolates were identified as *U. vitis-viniferae* and *U. yakutkhanika.* These species are described as follow:

Uzbekistanica vitis-viniferae Crous & Akulov, Fungal Systematics and Evolution 6: 225 (2020).

Conidiomata pycnidial, solitary, globose, dark brown, smooth, ostiolate. Conidiophores, hyaline, smooth, subcylindrical, branched, 1–2-septate, 17–23 × 2.5–3 µm. Conidiogenous cells in clusters of 2–6, phialidic, subcylindrical, 7– 9 × 2–2.5 µm. Conidia

'cytospora-like', aseptate, hyaline, smooth, subcylindrical with obtuse ends, straight to slightly curved, 4–5× 1.5 µm (Fig. 2).

Notes: The distinguishing feature of this species from other reported species is the different shape of conidia which is aseptate and 'cytospora-like'.

Host and distribution: On dead stem of *Vitis-viniferae* from Ukraine.

Specimens examined: SB435 (IRAN 4522C), IRAN, Kermanshah Province, pathogenic on twigs of *Juglans regia*, 16 May. 2018, S. Bagherabadi, Accession NO. OK560355.

Fig. 1. a-c. Natural symptoms of black spots caused by *Uzbekistanica* spp. on walnut trees; d. artificial symptoms after inoculation with SB425 isolate; e. artificial symptoms after inoculation with SB435 isolate on healthy shoots of walnut trees; f. negative control without any symptoms.

Uzbekistanica yakutkhanika Wanas, Gafforov & K.D.Hyde, Fungal Diversity 89: 102 (2018)

Conidiomata pycnidial, stromatic, solitary, globose, dark brown to black, ostiolate, apapillate. Conidiogenous cells, cylindrical to subcylindrical, hyaline, forming typical phialides with periclinal thickenings, swollen at the base, discrete, producing a single conidium at the apex.

Conidia 5–7 × 3.5–4 µm, initially hyaline, unicellular, becoming dark brown and 1-septate while still attached to conidiogenous cells; moderately thick-walled, wall externally smooth, ellipsoid to ovoid, widest in the center, base rounded (Fig. 3).

Notes: The distinguishing feature of this species from other reported species is in the shape of conidia which are ellipsoid to ovoid, widest in the center, base rounded and 1-septate while in *U. pruni*, conidia are oblong to ellipsoidal, with rounded ends, occasionally truncate base, initially hyaline, unicellular, 3–4-septate and not constricted at the septa.

Host and distribution: On branches of *Rosa hissarica*, Uzbekistan. Specimens examined: SB425 (IRAN 4521C), IRAN, Kermanshah Province, pathogenic on twigs of *Juglans regia*, 16 May. 2018, S. Bagherabadi, Accession NO. OK560354.

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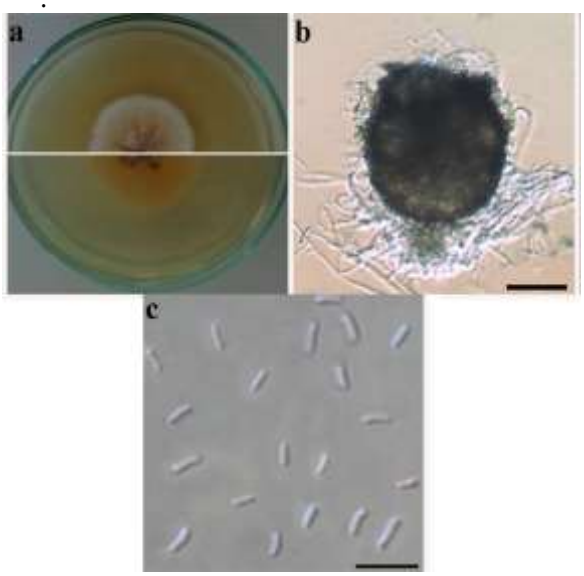

Fig. 2. *Uzbekistanica vitis-viniferae*. a. Surface of colony (top) and back side (down) on PDA; b-conidiomata — Scale bar = 50 µm; c. conidia — Scale bar = 10 µm.

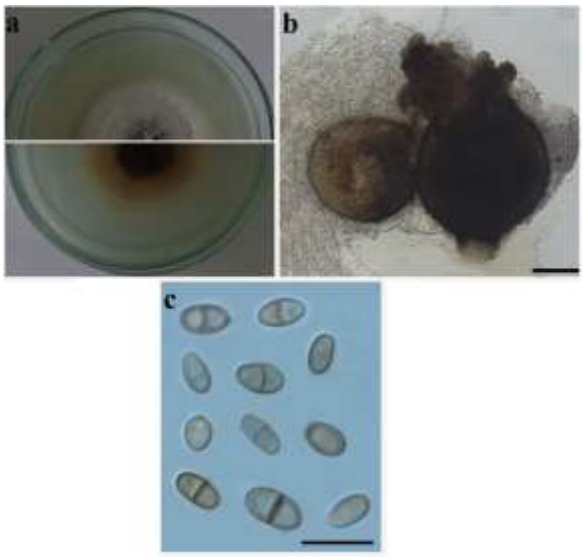

Fig. 3. *Uzbekistanica yakutkhanica.* a. Surface of colony (top) and back side (down) on PDA; b-conidiomata — Scale bar = 100 µm; c. conidia — Scale bar = 10 µm.

Phylogenetic analysis

BLASTn alignment on NCBI revealed that the ITS sequences for representative isolates had the highest similarity with the ex-type strain of *U. vitis-viniferae* and *U. yakutkhanica* in accordance with morphological identification. Maximum likelihood analyses indicated that representative isolate SB425 (IRAN 4521C) obtained from symptomatic twigs of walnut trees in this study clustered in a single

strongly supported clade that includes the ex-type isolates of *U. yakutkhanica* and the representative isolate SB435 (IRAN 4522C) obtained from

symptomatic twigs of walnut trees in this study clustered in a single strongly
supported clade that includes the ex-type isolates of *U. vitis-viniferae* available in the GenBank. Molecular assay by ML analyses confirmed the morphological identification and phylogenetic position of SB425 and SB435 isolates with reference isolates of *Uzbekistanica* spp. (Fig. 4).

Pathogenicity assay

The inoculated shoots were examined daily to explore the progress of the symptoms. The symptoms appeared on inoculated shoots after three weeks. No symptoms were observed on negative controls. *U. vitis-viniferae* and *U. yakutkhanica* were consistently re-isolated from inoculated shoots successfully. The results of pathogenicity tests indicated that these isolates were pathogenic on walnut shoots (Fig. 1).

DISCUSSION

In this study, 19 isolates were obtained from twigs with black spots symptoms of walnut trees in Hamedan and Kermanshah provinces of Iran. Although the surveys were performed from major walnut-growing areas in Iran during the years 2016-2020, but the prevalence of these symptoms were observed only in Hamedan and Kermanshah provinces between 2018 and 2019. However, it cannot be claimed that the presence of this species on walnut trees occurs only in these two provinces of Iran. After examination of cultural and morphological features, obtained isolates were grouped in two categories. Based on morphological studies, these two groups were identified as *U. vitis-viniferae* and *U. yakutkhanika* along with molecular analysis of two representative isolates.

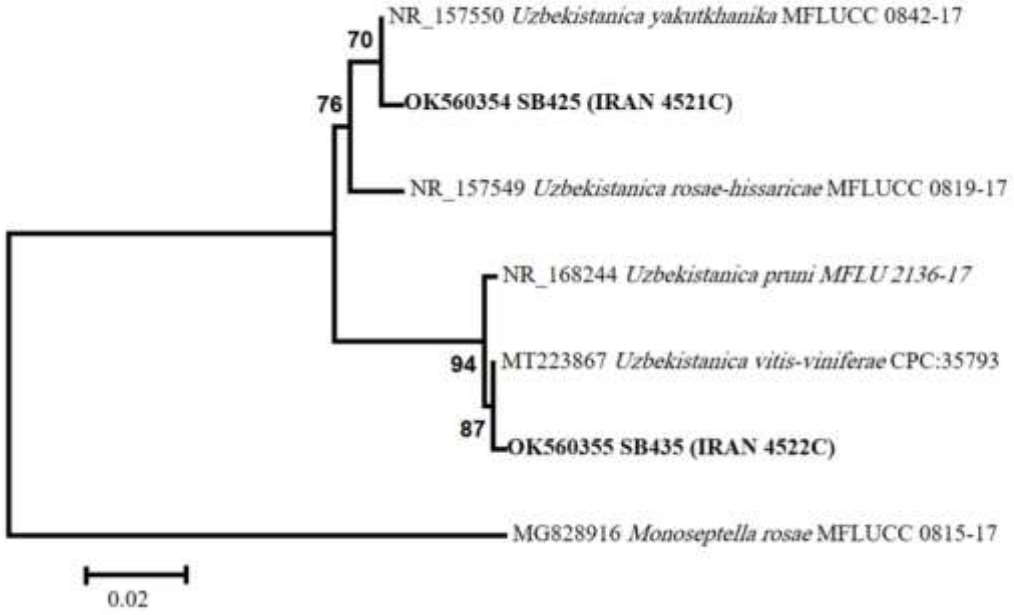

Fig. 4. Maximum Likelihood phylogenetic tree based on ITS sequences of *Uzbekistanica* spp. isolated from walnut trees in Iran and ex-type strains of *Uzbekistanica* from GenBank. Numbers at the nodes are the bootstrap values obtained for 1000 replicates. The analysis involved seven nucleotide sequences. There were a total of 539 positions in the final dataset. Isolates obtained in this study shown in bold and the tree is rooted to *Monoseptella rosae* MFLUCC 0815-17.

Phylogenetic analyses revealed that obtained isolates of Iran is closely related to ex-type isolates of *U. vitis-viniferae* and *U. yakutkhanika*, which recorded in the GenBank.

Reported species in this study were distinguished from other reported species based on morphological features and ITS analysis clearly. Pathogenicity examination of obtained isolates from Iran confirmed that these isolates are pathogenic on walnut shoots, however there is any report of pathogenicity of *Uzbekistanica* spp. It should be noted that the severity of the symptoms in *U. vitis-viniferae* was higher than *U. yakutkhanika* (Fig. 1 d-e). *Uzbekistanica vitis-viniferae* was previously reported on dead stem of *vitis-viniferae* from Ukraine (Wanasinghe et al. 2018) also *U. yakutkhanika* was reported on branches of *Rosa hissarica* Slobodov from Uzbekistan (Crous et al. 2020). Since there is not any report of *Uzbekistanica* on walnut trees in the world, it can be

considered that walnut tree is a new host for these species.

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شناسایی مورفولوژیکی و مولکولی گونه های *Uzbekistanica* جدا شده از درختان گردو

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چکیده: طی نمونه برداری در سال های ۱۳۹۷-۱۳۹۸، از باغ های گردو استان های همدان و کرمانشاه، نقاط سیاه رنگ روی شاخه های گردو مشاهده شد. به دلیل شیوع این علائم روی شاخه های درختان گردو، نمونه ها برای بررسی های بیشتر، به آزمایشگاه قارچ شناسی منتقل شد. پس از جداسازی و خالص سازی روی محیط PDA، ۱۹ جدایه از شاخه های دارای علائم به دست آمد. مشاهدات میکروسکوپی برای شناسایی های مورفولوژیکی و گروه بندی جدایه ها، انجام شد. براساس ویژگی های مورفولوژیکی، دو جدایه به عنوان نماینده برای شناسایی های مولکولی انتخاب شدند. شناسایی مولکولی براساس توالی یابی ناحیه ITS، نشان داد که این جدایه ها *Uzbekistanica vitis-viniferae* و *Uzbekistanica yakutkhanica* بودند. آزمایشات بیماری زایی در شرایط آزمایشگاهی، نشان داد که جدایه های به دست آمده از درختان گردو روی شاخه های سالم گردو بیماری زا بودند. براساس دانش ما، این اولین گزارش از *Uzbekistanica*، به عنوان جنس جدید برای فونگای ایران است. علاوه بر این، درخت گردو به عنوان میزبان جدیدی برای *U. vitis-viniferae* و *U. yakutkhanica* در جهان است.

کلمات کلیدی: *Juglans regia, Melanommataceae, Pleosporales,* ITS, *Uzbekistanica*

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