Morphological and molecular identification of a newly recovered *Pythium* species, *P. viniferum* from Iran, and evaluation of its pathogenicity on cucumber seedlings

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Key words: Biodiversity, *Cucumis sativus*, Mycobiota, *Pythium viniferum.* – Mycobiota of Iran.

Abstract: During a survey on the biodiversity of the genus *Pythium* in Iran, 12 isolates of *P. viniferum* were recovered from the rhizosphere of plant species from different locations and environments. The identification of *P. viniferum* was based on combination of cultural morphological characteristics, its cardinal *in vitro* growth rate, and sequence data from ITS-rDNA. Phylogenetic analyses of the ITS-rDNA sequences clustered our isolates with ex-type isolate of *P. viniferum* from GenBank. The species represents a new record for the mycobiota of Iran. We provide a full illustration of the species and compare its phylogeny and morphology with closely related species in *Pythium* clade F. Inoculation experiments indicated that it was capable of infection in cucumber (*Cucumis sativus*) seedlings.

Zusammenfassung: Im Rahmen einer Studie zur Biodiversität der Gattung *Pythium* im Iran wurden 12 Isolate von *P. viniferum* aus der Rhizosphäre von Pflanzenarten von verschiedenen Fundorten und Umweltbedingungen gewonnen. Die Identifizierung von *P. viniferum* basierte auf einer Kombination von kulturbezogenen morphologischen Merkmalen, seiner kardinalen in vitro Wachstumsrate und ITS-rDNA Sequenzdaten. Phylogenetische Analysen der ITS-rDNA-Sequenzen gruppierten unsere Isolate mit dem Ex-Typus-Isolat von *P. viniferum* von GenBank. Die Art ist ein neuer Nachweis für die Mykobiota des Iran. Wir geben eine vollständige Darstellung der Art und vergleichen ihre Phylogenie und Morphologie mit nahe verwandten Arten in *Pythium* Klade F. Inokulationsexperimente zeigten, dass die Art in der Lage war, Gurken (*Cucumis sativus*)-Sämlinge zu infizieren.

The genus *Pythium* is a complex and highly diverse genus, containing more than 140 species and extending to different environments e.g., terrestrial and aquatic habitats (KIRK & al. 2008). Some species of *Pythium* exhibit saprotrophic and parasitic life styles (LARA & BELBAHRI 2011). Parasitic species of the genus may have negative effects on wide range of hosts including algae, plants, insect larvae, animals and human (MIURA & al. 2010, PHILLIPS & al. 2008, VAN DER PLAATS-NITERINK 1981).

Some of plant pathogenic species have a damaging impact on crops throughout the world, causing pre- and post-emergence damping-off and seed and root rot. Some species in the genus can be beneficial, functioning as biocontrol agents that protect against other pathogenic *Pythium* species and other fungi (PAUL 2004). The mainland of Iran covers diverse climatic zones with a great biodiversity of plants and accordingly a high diversity of fungi. The exploration of oomycete species is, however, far from being complete and mostly based on morphological features. From past to now, 43 species, four species groups and one unknown species of *Pythium* were recorded on different plants in Iran (ERSHAD 1977; MOSTOWFIZADEH–GHALAMFARSA & BANIHASHEMI 2005; CHENARI BOUKET & al. 2015, 2016). During this study as part of a comprehensive survey to ascertain the species diversity of *Pythium* in Iran, isolates of the genus, later identified as *P. viniferum*, were recovered from the rhizosphere of plants of *Alyssum* sp., *Conringia orientalis, Malus domestica*, *Malva silvestris*, *Prunus amygdalinum*, *P. cerasus, Prunus* sp., *Sisymbrium loeselii*, *Sphagnum* sp. and *Stellaria media* from different locations of northwestern Iran. We evaluated pathogenicity of our ex-type isolate (OPU 1675) of *P. viniferum* on cucumber (*Cucumis sativus*) seedlings under greenhouse conditions.

Material and methods

Isolation: Isolations were done by excising short pieces of roots of these plants that were washed twice in sterile distilled water and then dried on a sterile paper towel, transferred onto oomycete–selective media namely NARM and NARF (MORITA & TOJO 2007; CHENARI BOUKET & al. 2015, 2016) and incubated at 25 °C for 1–2 days. Pure cultures were established using a hyphal tip technique. Cultures were preserved on V8A (MILLER 1955) slant vials at 10 °C in the dark until used. Collection locations are provided in Tab. 1.

Morphology: Mycelial patterns of all isolates were recorded in 9-cm-diam. plates, two days after inoculation in the dark at 22 °C on CMA (Becton Dickinson and Company, Franklin Lakes, NJ, USA), PDA (Sigma Aldrich, St. Louis, MO, USA) and V8A (MILLER 1955). Morphological observations were made on structures produced on PCA media and sterile grass blades floated in sterile pond water, distilled water and tap water (MARTIN 1992). Twenty measurements were made for each microscopic structure including main hyphae, sporangia (hyphal-swellings), antheridia, oogonia and oospores. Photographs were captured using an Olympus-BX43 microscope with the digital camera system (DP2-ASL) (Olympus, Tokyo, Japan). The cultures were deposited in the Culture Collection of Osaka Prefecture University (OPU), Osaka, Japan. The cardinal temperatures were determined 1 day after inoculation on PCA at temperatures between 4 to 43 °C.

Molecular phylogeny: DNA was extracted from mycelia grown on CMA with a manual process described by Moller & al. (1992). The internal transcribed spacer (ITS) of ribosomal DNA was amplified by the polymerase chain reaction using primers ITS5 (GGAAGTAAAGTCGTAACAAGG) and ITS4 (TCCTCCGCTTATTGATATGC) (WHITE & al. 1990). All reactions were carried out in a total volume of 50 µl, containing 5 µl 10X Taq buffer (20 mM Tris-HCl, pH 8.0, 100 mM KCl), 4 µl 2.5 mM dNTP mixture, 0.5 µM of each primer, 1.25 units Taq DNA polymerase (Takara Bio, Ohtsu, Japan) and 10 ng DNA. The amplifications were performed using a PerkinElmer 9700 thermal cycler (Perkin Elmer Inc. Waltham, MA, USA) with the following cycling profile: 95 °C for 5 min followed by 30 cycles including denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 1 min, and final extension step at 72 °C for 7 min. PCR amplicons were purified with GenElute™ PCR Clean–Up Kit (Sigma-Aldrich, St. Louis, MN, USA) based on the instructions of the manufacturer and then used for sequencing. The amplicons were sequenced in both directions using the same PCR primers and the BigDye® Terminator v. 3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) on the basis of the manufacturer's manual and then analyzed on 3130x Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Raw sequence files were manually edited

using SeqMan™II (DNASTAR, Madison, WI, USA) and a consensus sequence was generated for forward-reverse sequences of each isolate. The consensus sequence was compared against the NCBI's GenBank sequence database using Megablast to identify their closest matches. The sequences retrieved from GenBank (including *Pythium* clade F species) together with sequences generated in this study, were aligned using MEGA v. 6 (TAMURA & al. 2013). For phylogenetic comparison, Bayesian inference analysis was conducted with MrBayes v. 3.2.1 (RONQUIST & HUELSENBECK 2003). The best evolutionary model for data partition was obtained using MrModelTest v. 2.3 (NYLANDER 2004). The heating parameter was set at 0.15 and four Markov chains were run for 10000000 generations, sampling every 1000 steps, and with a burn in at 25 % sampled trees until the average standard deviation of split frequencies was below 0.01. The resulting phylogenetic tree was exported with Geneious v. 5.6.7 (DRUMMOND & al. 2012). Sequences derived from this study were deposited at NCBI's GenBank nucleotide database (http://www. ncbi.nlm.nih.gov; Tab. 1).

Pathogenicity assay: A pot inoculation assay was conducted to evaluate the ability of *P. viniferum* (isolate OPU 1675) to cause damping-off. CMA plugs containing living mycelia of *P. viniferum* were transferred to a 300 ml flask containing 3 g of autoclaved seeds of *Festuca arundinacea* and 12 ml of distilled water. After two weeks of incubation at 22 °C in darkness, 15 g of these colonized seeds were mixed with 3 kg of commercial nursery soil (Aisai-1, Katakura Chikkarin, Tokyo, Japan) with a mortar. Infested soil (90 ml) was placed in each of five pots and 10 seeds of cucumber (*Cucumis sativus* L. cv. 'Aonagakei-Jibai', Takii & Co. Ltd., Kyoto, Japan) were in-serted in the soil. The seeds were covered with 30 ml of the infested soil was placed on top. Pots with seeds planted in non–infested soil were used as control. Each pot was irrigated immediately then every day, and incubated at 22 °C under continuous light conditions (80 µmol $m^{-2}s^{-1}$) in a growth chamber. The percentage of diseased (with damping-off symptoms) plants and healthy plants was recorded after 12 days (data not shown). The experiment was repeated three times.

Results

Morphology: Cultures of *Pythium* isolate OPU 1675 growing on CMA, V8A and PDA exhibited submerged mycelium with weak to radiate growth of aerial mycelium. Optimum growth occurred at 22 °C on PCA, with the average daily growth rate of 25 mm. The minimum, optimum and maximum growth temperature on PCA were 7, 22 and 35 °C, respectively. The main hyphae were hyaline and up to 7 µm in diameter and hyphal swellings were germinated by 1–4 germ tubes. Appressoria were simple or catenulate, curved or sickle–shaped with sexual structures occasionally developing on them. Oogonia were smooth, globose, terminal or intercalary, 21–25 µm in diam. Antheridia were 1–2 per oogonium, monoclinous and diclinous. Oospores were plerotic, 17–23 µm in diam. Rarely, two oospores were formed within one oogonium (Fig. 1). Cultural and morphological features of isolate OPU 1675 were identical in every respect to those of *P. viniferum* provided by PAUL & al. (2008).

DNA phylogeny: 12 isolates of *Pythium* suspected of being *P. viniferum* were subjected to DNA sequence analyses. The final aligned ITS dataset contained 68 ingroup taxa with a total of 317 characters, containing 44 unique site patterns. *Pythium ultimum* (GenBank accession HQ643877) served as the outgroup taxon. The heating parameter was set to 0.15. The results of MrModeltest recommended JC+I+G models with a gamma distributed rate variation and dirichlet base frequencies. During the generation of the ITS tree, a total of 1242 trees were saved, and consensus trees and poste-

Tab. 1. Code, GenBank accession numbers, locality and references of *Pythium* strains used in the phylogenetic analysis.

Species	Code	Locality	ITS GenBank Acc. No.	Reference
P. viniferum	OPU 1664	Tabriz, East-Azarbaijan province (38° 04′ N, 46° 18′ E)	**KU743387**	**Present study**
P. viniferum	OPU 1665	Tabriz, East-Azarbaijan province (38° 04′ N, 46° 11′ E)	**KU743388**	**Present study**
P. viniferum	OPU 1666	Ahar, East-Azarbaijan province (38° 28′ 39″ N, 47° 04′ 12″ E)	**KU743389**	**Present study**
P. viniferum	OPU 1667	Parsabad, Ardebil province (39° 38′ 54″ N, 47° 55′ 03″ E)	**KU743390**	**Present study**
P. viniferum	OPU 1670	Meshkin Shahr, Ardebil province (38° 23′ 56″ N, 47° 40′ 55″ E)	**KU743391**	**Present study**
P. viniferum	OPU 1671	Meshkin Shahr, Ardebil province (38° 23′ 56″ N, 47° 40′ 55″ E)	**KU743392**	**Present study**
P. viniferum	OPU 1672	Ahar, East-Azarbaijan province (38° 51′ 33″ N, 47° 22′ 07″ E)	**KU743393**	**Present study**
P. viniferum	OPU 1673	Ardebil, Ardebil province (38° 15′ N, 48° 20′ E)	**KU743394**	**Present study**
P. viniferum	OPU 1675	Kandovan, East-Azarbaijan province (37° 47′ 42″ N, 46° 14′ 55″ E)	**KU743395**	**Present study** **Present study**
P. viniferum	OPU 1677	Ajabshir, East-Azarbaijan province (37° 28′ 47.4″ N, 45° 54′ 18″ E)	**KU743396**	
P. viniferum	OPU 1678	Marand, East-Azarbaijan (38° 27′ 05″ N, 45° 47′ 10″ E)	**KU743397**	**Present study**
P. viniferum	OPU 1679	Maragheh, East-Azarbaijan (37° 23′ 51.5″ N, 46° 14′ 00″ E)	**KU743398**	**Present study**
P. viniferum (original ex–type)	F-1201	France	AY455694	PAUL & al. 2008
P. attrantheridium	DAOM230387	United States	HQ643475	ROBIDEAU & al. 2011
P. attrantheridium	DAOM230383	Canada	HQ643477	ROBIDEAU & al. 2011
P. intermedium	BR1042	Canada	HQ643579	ROBIDEAU & al. 2011
P. intermedium	BR128	Canada	HQ643578	ROBIDEAU & al. 2011
P. intermedium	BR339	Canada	HQ643577	ROBIDEAU & al. 2011
P. intermedium	BR485	Netherlands	HQ643576	ROBIDEAU & al. 2011
P. paroecandrum	BR601	Australia	HQ643735	ROBIDEAU & al. 2011

Tab. 1. Continued.

Species	Code	Locality	ITS GenBank Acc. No.	Reference
P. paroecandrum	BR773	South Africa	HQ643734	ROBIDEAU & al. 2011
P. paroecandrum	BR774	South Africa	HQ643733	ROBIDEAU & al. 2011
P. paroecandrum	BR807	South Africa	HQ643732	ROBIDEAU & al. 2011
P. paroecandrum	BR929	Spain	HQ643730	ROBIDEAU & al. 2011
P. paroecandrum	CBS15764	Australia	HQ643731	ROBIDEAU & al. 2011
P. sylvaticum	BR1045	Canada	HQ643852	ROBIDEAU & al. 2011
P. sylvaticum	BR1069	Canada	HQ643851	ROBIDEAU & al. 2011
P. sylvaticum	BR171	Canada	HQ643850	ROBIDEAU & al. 2011
P. sylvaticum	BR179	Canada	HQ643849	ROBIDEAU & al. 2011
P. sylvaticum	BR599	Canada	HQ643848	ROBIDEAU & al. 2011
P. sylvaticum	P15580	Canada	HQ261741	ROBIDEAU & al. 2011
P. sylvaticum	CBS45367	United States	HQ643845	ROBIDEAU & al. 2011
P. sylvaticum	BR647	Netherlands	HQ643847	ROBIDEAU & al. 2011
P. sylvaticum	Lev1544	United States	HQ643846	ROBIDEAU & al. 2011
P. terrestris	ADC9906	Netherlands	HQ643858	ROBIDEAU & al. 2011
P. terrestris	BR922	United States	HQ643856	ROBIDEAU & al. 2011
P. terrestris	CBS112352	France	HQ643857	ROBIDEAU & al. 2011
P. mamillatum	BR648	Netherlands	HQ643689	ROBIDEAU & al. 2011
P. mamillatum	BR765	Canada	HQ643688	ROBIDEAU & al. 2011
P. mamillatum	CBS25128	Netherlands	HQ643687	ROBIDEAU & al. 2011
P. spiculum	CBS122645	France	HQ643790	ROBIDEAU & al. 2011
P. kunmingense	CBS55088	China	HQ643672	ROBIDEAU & al. 2011
P. spinosum	CBS27667	Netherlands	HQ643792	ROBIDEAU & al. 2011
P. spinosum	Lev1526	United States	HQ643794	ROBIDEAU & al. 2011
P. spinosum	CBS122663	India	HQ643791	ROBIDEAU & al. 2011
P. macrosporum	BR1029	Canada	HQ643685	ROBIDEAU & al. 2011
P. macrosporum	ADC0029	Norway	HQ643686	ROBIDEAU & al. 2011
P. macrosporum	CBS57480	Netherlands	HQ643684	ROBIDEAU & al. 2011
P. emineosum	BR836	Canada	GQ244428	BALA & al. 2010
P. emineosum	BR479	Canada	GQ244427	BALA & al. 2010
P. intermedium	BR707	Canada	HQ643575	ROBIDEAU & al. 2011
P. ultimum	BR1036	Canada	HQ643877	ROBIDEAU & al. 2011

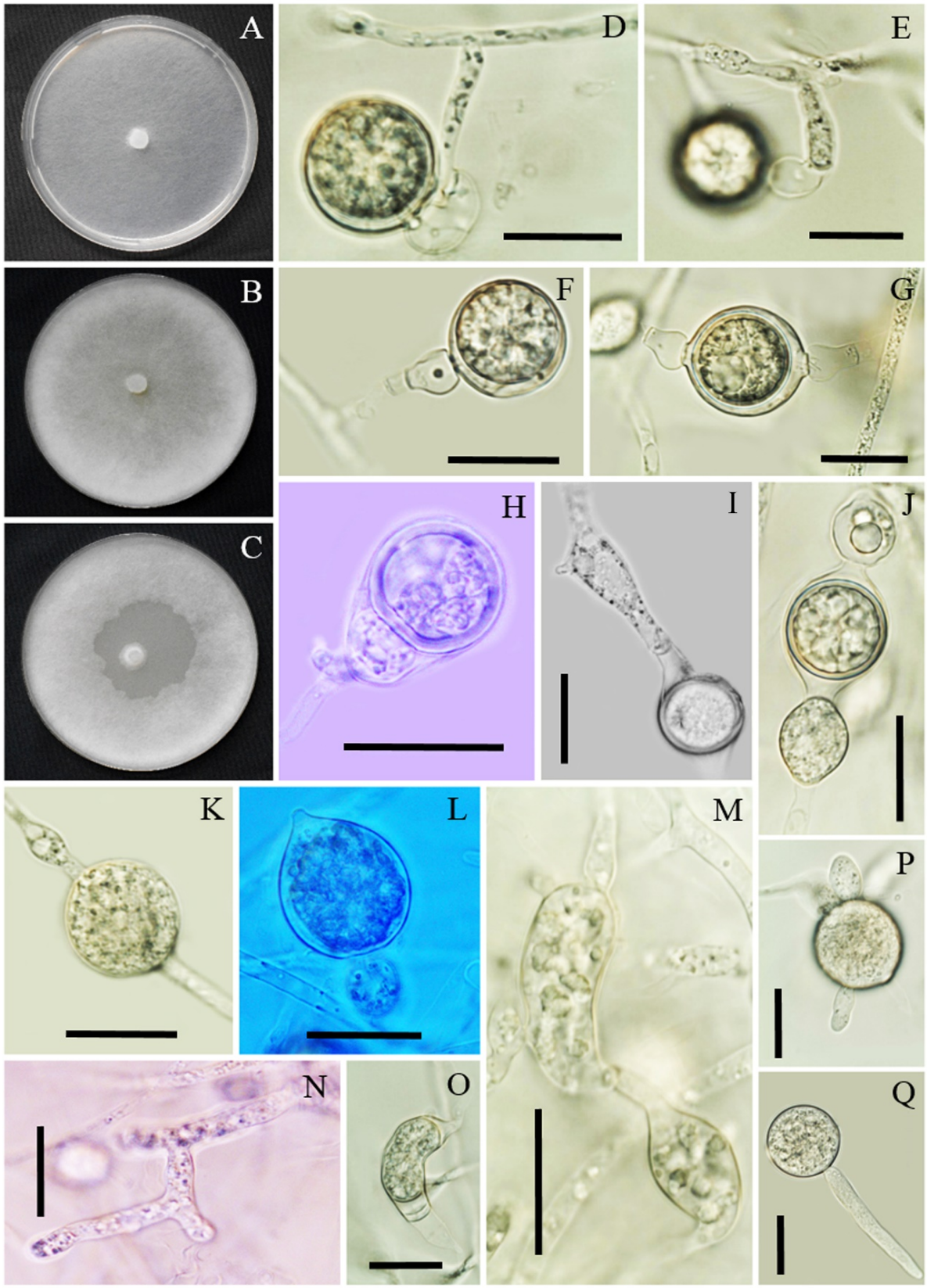

Fig. 1. *Pythium viniferum* (A–Q). Colony morphology of the isolate OPU 1675 on CMA (A), PDA (B), V8A (C), oogonium with a monoclinous antheridium (D–F), oogonium with monoclinous and diclinous antheridia (G), oogonium with two oospores (H), terminal plerotic oospore developing from an appressorium (I), intercalary plerotic oospores (J), non–proliferating hyphal swellings (K–L), appressoria (M–O), Hyphal swellings germinating by 1–4 germ tubes (P, Q). Bars: 20 µm.

Fig. 2. Consensus phylogram (50 % majority rule) of 932 trees resulting from a Bayesian analysis of ITS sequence alignment using MrBayes v.3.2.1. The scale bar indicates 0.01 expected changes per site. Numbers above/below the branches represent posterior probabilities. The tree was rooted to *Pythium ultimum*.

rior probabilities were calculated from the remaining 932 (75 %) trees. Based on the result of the ITS sequence data, isolates of *P. viniferum* obtained in this study grouped in the separate clade together with the original ex-type of *P. viniferum* from GenBank (Fig. 2).

Morphological characteristics and molecular studies indicate that the isolates from the plant species mentioned above are *P. viniferum*. This is the first report of the species on plants in Iran.

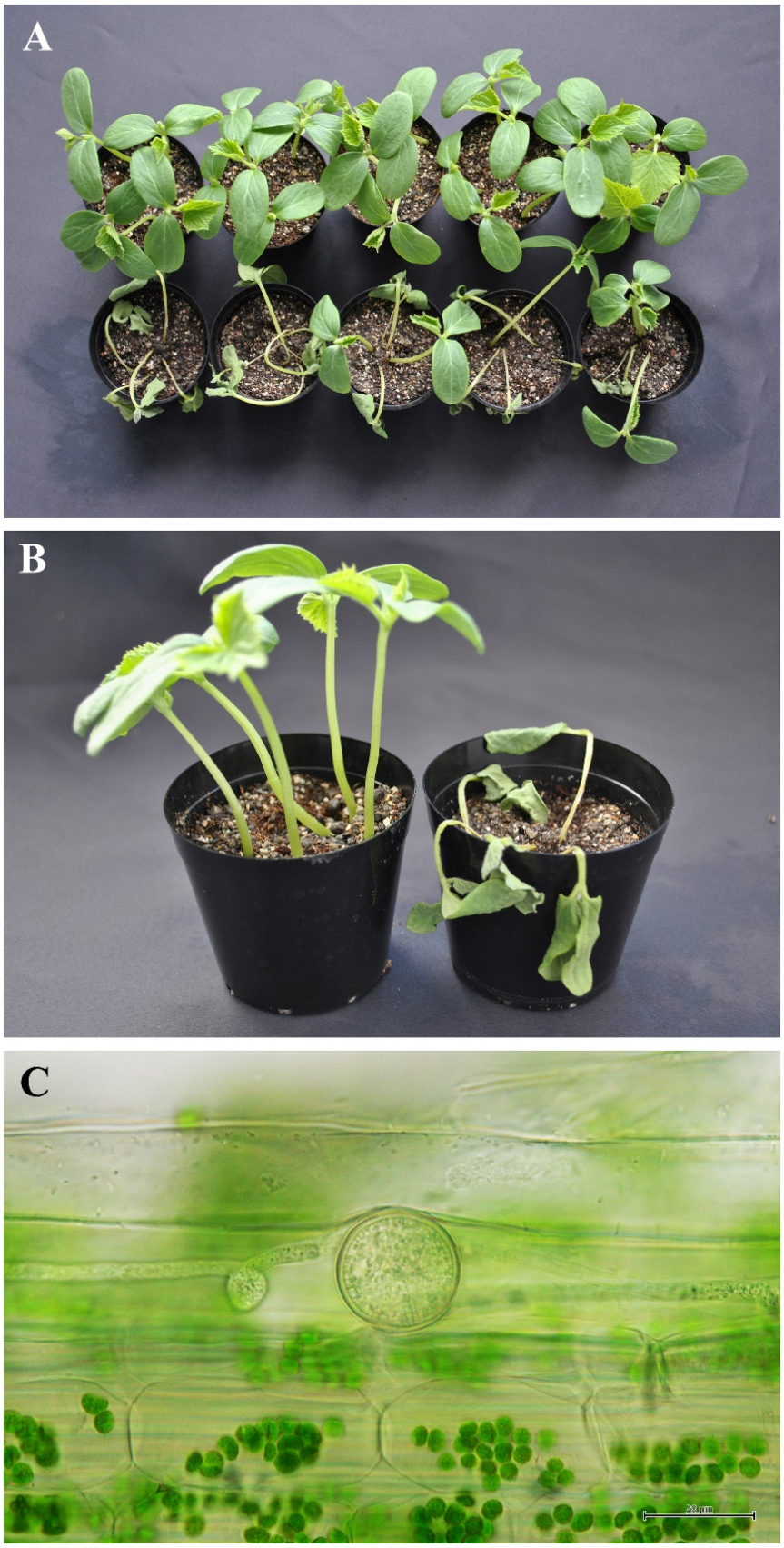

Fig. 3. Pathogenicity of *P. viniferum* on cucumber seedlings. Healthy control plants (upper row A, and left B) and collapsed plants inoculated with *P. viniferum* isolate OPU 1675 (lower row A, and right B). Oospores of *P. viniferum* (C) formed inside stem cells of *Cucumis sativus*. Bar: 20 µm.

Tab. 2. Morphological comparison between *Pythium viniferum* (OPU 1675) and its morphologically and phylogenetically related species.

haracteristics	*Pythium* OPU 1675	*P. lucens* (ALI-SHTAYEH & DICK 1985)	*P. abappressorium* (PAULITZ & al. 2003)
Main hyphae	>5 µm	3.5–6.5 µm	>5 µm
Sporangia	Produced as non-sporulating hyphal swellings; Zoospores not formed	Globose or subglobose, terminal and occasionally catenulate 2–3 in a chain, intercalary (21–25 µm); Discharge tube up to 30 µm long, 1–2 per sporangium; Encysted zoospores 8–10 µm in diam.	Globose, (11–)16–22(–30) µm, terminal or intercalary or formed from appressoria; remains of appressorium often attached to the base of sporangium. Zoospores formed at 20°C discharging with exit tubes 2–4 µm long
Hyphal swellings	Germinating by 1–4 germ tubes	Not formed	Globose, lemon–shaped, or cylindrical, terminal or intercalary in hyphae or appressorium, (11–)13–22(–24) µm long, (8–)10–18(–20) µm wide
Appressoria	Simple or catenulate, curved or sickle-shaped	Not formed	Up to 160 µm long, 8–12 µm wide. Appressoria curved to sickle-shaped, often branched or in chains
Oogonia	Smooth, globose, terminal or intercalary, 21–25 µm in diam., sometimes arise from appressoria	Smooth–walled, globose, 22–35 µm in diam. rarely pyriform, usually terminal, occasionally intercalary	Smooth, terminal or produced within hyphae or appressoria
Antheridia	1–2 per oogonium, monoclinous and diclinous	1–2(–5) per oogonium, monoclinous, usually stalked, originating usually more than 20 µm distance from the oogonium base, occasionally diclinous, antheridial cells clavate, occasionally 2–3 borne on one antheridial branch	1–3 per oogonium, sac-shaped to crook-necked, 7–15 µm long, 4–9 µm wide, mostly monoclinous, occasionally hypogenous
Oospores	Plerotic, 17–23 µm in diam. Rarely, 2 oospores were formed within one oogonium	Aplerotic, Usually single, occasionally 2 oospores per oogonium, globose, 17 – 23 µm in diam.	Plerotic, smooth, globose, (12–)14–17(–27) µm diam., 1 or occasionally 2 per oogonium
Daily growth rate on PCA	25 mm/day	9 mm/day	15 mm/day

Pathogenicity: *Pythium* isolate OPU 1675 was pathogenic on cucumber seedlings. Symptoms initially appeared as small water-soaked lesions at the base of the stems that later coalesced and caused the stems to weaken and rot. Oogonia and oospores were found inside the stems of inoculated seedlings. Most (89.3 %) of the seedlings died and *P. viniferum* was isolated from all infected plants (Fig. 3).

Discussion

Pythium Clade F (sensu LEVESQUE & DE COCK 2004) consists of highly diverse and important plant pathogens. Most species do not or rarely produce zoospores. They produce either globose, non-proliferating sporangia or globose hyphal swellings and have fast growth and moderate cardinal temperatures (LEVESQUE & DECOCK 2004). The species was originally isolated from soil in the Burgundian vineyards of France (PAUL & al. 2008). Based on the results of the ITS-rDNA sequence data, all *P. viniferum* sequences obtained in this study nested within *Pythium* clade F species. This species can be distinguished morphologically and phylogenetically from its closest relatives, such as *P. abappressorium* and *P. lucens*, by its sexual structures that may originate from appressoria, smaller oogonia and inability to produce zoospores (Tab. 2). Regarding previous studies, all plants on which we found *P. viniferum* are new substrates associated with this species and this is the first record of *P. viniferum* on these hosts in the world.

Cucumber is one of the most sensitive hosts for *Pythium*. The plant has been commonly used as an indicator plant for weak- or non-pathogenic species of *Pythium* (VAN DER PLAATS-NITERINK 1981). *Pythium* spp. are opportunistic plant pathogens. Therefore, it is important to know their potential to cause disease at least on the sensitive crops. Cucumber is one of the major greenhouse vegetable products throughout the world and especially in Iran. It is a warm-season plant and grows well at moderate temperatures (24–29 °C). It is cultivated on 78000 ha and the production is more than 1.72 million tonnes. Recently, greenhouse areas of Iran increased from 3380 ha to 6630 ha at an increasing rate of 96 % (MOHAMMADI & OMID 2010). We found that Iranian isolates of the species were pathogenic on cucumber seedlings. Therefore, the species can be a potential threat to the cultivation of vegetables in glasshouses and some sensitive crops.

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