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New report of *Phytophthora occultans* associated with root and crown rot on Sansevieria

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Abstract: In the summer of 2022, sansevieria plants with symptoms of root and crown rots were detected in greenhouses of Isfahan province, Iran. Symptomatic tissues of the root were cultured on CMA-PARPH. Recovered oomycete isolates were purified by single-zoospore technique on WA. The baiting method using citrus leaf disc was also used to isolate *Phytophthora* spp. isolates from the soil around roots. Four isolates were obtained directly from infected root tissues and two isolates from soil. Fungal isolates were identified based on morphological characteristics and molecular data of *β*-tubulin (*βtub*) and translation elongation factor 1-alpha gene (*tef1*-α) genes. According to the morphological and phylogenetic analysis, all isolates were recognized as *Phytophthora occultans*. Koch's postulates were completed and confirmed that *Phytophthora occultans* isolates were responsible for sansevieria root and crown rots. To our knowledge, this is the first report that *Phytophthora occultans* associated with root rot of sansevieria in Iran and probably in the world.

Key words: *Dracaena trifasciata*, *β*-tubulin**,** *tef1-α*, Iran, Isfahan**,** *Oomycota*, Sansevieria

INTRODUCTION

Sansevieria (*Dracaena trifasciata* (Prain) Mabb.) is a genus of flowering plants that belonged to the family *Asparagaceae*. It is known as snake plant or mother-in-law's tongue due to its leaf shape and margin sharpness. This plant is native to tropical Africa and was introduced to America, Asia, Australia, and the Pacific Islands as an ornamental and fiber crop (USDA-ARS 2017). In Iran, sansevieria is cultivated in greenhouses and is commonly used as an ornamental plant in offices and homes.

Several plant pathogens are reported to cause root and crown rots and leaf spots on sansevieria worldwide; leaf spot caused by some fungi such as *Neoscytalidium dimidiatum* (Penz.) Crous & Slippers (Kee *et al.* 2017; Monteles *et al.* 2020), *Stemphylium vesicarium* (Wallr.) E.G. Simmons (Ahmadpour and Poursafar 2018), *Curvularia* spp. (Kee *et al.* 2020a), *Stemphylium lycopersici* (Enjoji) W. Yamam (Kee *et al.* 2017), *Chaetomella* sp. (Li *et al.* 2013), *Colletotricum sansevieriae* M. Nakamura & M. Ohzono (Karimi *et al.* 2017; Kee *et al.* 2020b), *Lasiodiplodia* spp. (Kee *et al.* 2019), *Ascochyta citri* Penz., Michelia (Ershad 2022) *Colletotrichum neosansevieriae* Crous & N.A. van der Merwe and *Stachybotrys sansevieriicola* (Crous & M.J. Wingf.) L. Lombard & Crous (Crous et al. 2015), and root and crown rots caused by other fungi and fungal-like pathogens such as *Fusarium solani* (Mart.) Sacc., Michelia (Park *et al.* 2020), *Pythium spinosum* **(**Sawada) Uzuhashi (Takeuchi *et al.* 2002), and *Phytophthora nicotianae* Breda de Haan (Patel *et al.* 2016).

Phytophthora species are among the most destructive plant pathogens infecting broad range of plants, including forest trees, oil crops, vegetables, fruit, and ornamental plants worldwide (Martin et al. 2012). *Phytophthora* diseases are responsible for billions of dollars in yield and quality losses (Kamoun et al. 2015). Species of *Phytophthora* are frequently isolated from different ornamental plants in greenhouses and landscapes (Parke *et al.* 2014; Schlenzig *et al.* 2015). For example, *P. nicotianae* can infect over 200 genera in 90 families and usually exist in nurseries worldwide (Cline *et al.* 2008). In general, the infection process of *Phytophthora* species depends on humidity, which favors germination and dispersal of zoospores. Global distribution of *Phytophthora* species is believed to be through the commercial trade of ornamentals (Bienapfl and Balci 2014). For example, since the first detection of *P. ramorum* Werres, De Cock & Man in the 1990s, it has spread to Canada and the United States through shipments of infested ornamental plants (Prospero *et al.* 2009).

Sometimes, morphological features cannot provide all the essential data needed to describe a

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species. Currently, molecular methods have resolved many deficiencies of morphological identification and help accurately identify *Phytophthora* spp. (Puglisi *et al.* 2017; Jung et al. 2017; Martin et al. 2014). Thus, parts of the *Phytophthora* genome are used for phylogenetic-based taxonomy, including nuclear heat shock protein 90 (HSP90) (Jung *et al.* 2017), *βtub* gene (Maseko *et al*. 2007), translation elongation factor 1-alpha gene (*tef1-α*), cytochrome oxidase c genes (Puglisi *et al.* 2017; Ruano-Rosa *et al.* 2018), ITS-rDNA regions (Blair *et al*. 2008), 28S rDNA region (Yang et al. 2017), and *NADH1* gene (Jung *et al*. 2017).

In the summer of 2022, root and crown rots were observed on sansevieria in greenhouses of Isfahan Province, Iran. There are only reports of leaf spots caused by some fungi such as *Stemphylium vesicarium* and *Ascohyta citri* on sansevieria plants from Iran (Ahmadpour & Poursafar 2018; Ershad 2022). This research was conducted to determine the fungal and fungal-like pathogens causing root and crown rots on sansevieria based on morphological features and molecular confirmation using phylogenetic analyses of the DNA sequence data from β-tubulin (*βtub*) and translation elongation factor 1-alpha (*tef1*-α) genes.

MATERIALS AND METHODS

Sampling

In the summer of 2022, root and crown rots were observed on sansevieria in greenhouses of Isfahan Province, Iran. Symptomatic tissues were collected and taken to the laboratory in polyethylene bags in cool conditions and kept at 4 °C.

Fungal isolation of plant tissues

The infected roots were primarily separated, and rinsed with tap water for 2 hours to remove soil particles and saprophytes, cut into approximately 10 × 3-5 mm pieces, dried on sterile paper towels without extra treatments (no disinfection), and cultured on a semi-selective medium, CMA-PARPH (CMA: 40 g L^{-1} ground corn extract and 15 g L^{-1} agar amended with 200 µg ml^{-1} ampicillin, 10 µg ml^{-1} pimaricin, 25 µg ml^{-1} PCNB, 10 µg l^{-1} rifampicin and 50 µg ml^{-1} hymexazol) (Jeffers & Martin, 1986; Ghaderi & Banihashemi 2006). Plates were incubated at 25°±1C for five to ten days. Purification of isolates was performed using the single-zoospore technique on WA medium described in detail by Ghaderi and Habibi (2021).

Fungal isolation with baiting method

A baiting method using citrus leaf discs was used to isolate *Phytophthora* spp. from soil (Kannwischer & Mitchell 1978; Banihashemi & Ghaderi, 2006). In order to obtain *Phytophthora* species, 20 g of soil around the roots of sansevieria was placed into a round plastic container containing distilled water. Twenty lemon leaf disks, 5-8 mm in diam, were floated per container and incubated at 25 °C. After 48 hours, the baits were collected, washed with running tap water, blotted dry with a paper towel, and plated on CMA-PARPH medium. With the emergence of the fungus colonies from the baits, some boiled hemp seeds were placed on colonies of *Phytophthora* spp. isolates for 12-24 hours, then transferred into sterile distilled water in plastic Petri dishes and incubated at 25°C under continuous fluorescent illumination. Sporangia formation around the colonized hemp seeds gave a positive indication of *Phytophthora* spp. occurrence at the genus level.

Morphological characterization

Morphological identification was based on colony pattern, colony color, formation of hyphal swelling, growth rate on different media, and growth temperatures, morphology of sporangium, oogonium, antheridium, oospores, and chlamydospores according to the taxonomic keys of the *Phytophthora* species (Erwin & Ribeiro 1996; Gallegly & Hong 2008; Stamps *et al.* 1990).

Chlamydospore production was determined on solid media including CMA, PDA (potato dextrose agar), HSA (hemp seed agar), CA (carrot agar) and WA (carrot agar); on liquid media including CM, HS, PD, carrot broth and sterile soil extract (Mirsolemani & Mostowfizadeh-Ghalamfarsa 2013)

To identify *Phytophthora* species, it is necessary to see if an isolate is heterothallic or homothallic. Isolates were paired with themselves and with A1 and A2 mating types tester isolates (Shiraz University culture collection, Iran). Cultures were incubated at 18°±1 °C for one month in the dark. For morphological assay of the species, fifty sporangia, chlamydospores, oospores, oogonium, antheridium, and hyphae of each isolate were measured with Olympus light microscope CX31, equipped with a Dino-eye microscope eyepiece camera in conjunction with Dino Capture version 2.0 software.

Molecular Identification

Isolates were obtained from one-week-old pure culture on CMA media, and then a 5 mm plug was transferred into V8 broth on an orbital shaker at 20 °C for 7 days. Mycelia were harvested, washed with sterile distilled water and powdered in a sterile mortar using liquid nitrogen. Genomic DNA was extracted with the CTAB protocol defined in detail by Murray and Thompson (1980). The quality and quantity of the extracted DNA were evaluated on 1.2% agarose gel, viewed by staining in ethidium bromide, and the DNA samples were stored at -20 °C.

Molecular identification was performed using partial sequences of *tef1-α* gene (EF1/EF2, Blair et al. 2008) and *βtub* gene (TUBUF2 / TUBUR1, Kroon et al. 2004). PCR amplifications were performed in a final volume of 20 µl comprising 0.05 µM of each primer (CinnaGen, Iran), 0.4 µM dNTPs (MBI Fermentas), 1× Dream Taq Buffer (MBI Fermentas) and 0.5 U DNA Taq polymerase (MBI Fermentas). PCRs were performed in a Biometra thermocycler based on a protocol that was described in detail by Ghaderi and Habibi 2021. The quality of PCR products was checked on 1.2 % agarose gel with ethidium bromide solution and visualized by

VersaDoc Gel Imaging System (GelDoc, Bio-Rad Laboratories). PCR products were purified from agarose gels using a PCR purification kit (Fermentas) and DNA sequencing was performed in both directions by the DNA Sequencing Service of Macrogen Co. (South Korea).

The nucleotide sequences of *βtub* and *tef1-α* genes were blasted using Megablast to identify their closest neighbors, edited by BioEdit v. 7.2.5 (Hall 2012) and aligned using MAFFT v.7 (Katoh & Standley 2013). Manual adjustment of sequence alignments was performed to accommodate insertions/deletions. Phylogenetic analyses were performed in MEGA5 (Tamura et al. 2011) using the Maximum Likelihood method (Saitou & Nei 1987). *Phytophthora infestans* was used as an outgroup in the analysis. The obtained nucleotide sequences were deposited in GenBank (http://www.ncbi.nlm.nih.gov/), and all sequences used in this research are listed in Table 1.

Pathogenicity tests

Obtained isolates Sans-Po1 to Sans-Po6 were tested for their ability to cause symptoms on sansevieria plants in plastic pots. To produce inoculum, 200 ml of vermiculite and 120 mL hemp seed extract (60 g/L) were autoclaved, inoculated with a 6 mm CMA plug from actively growing hyphae of *Phytophthora* isolates, and incubated at room temperature for four weeks (Banihashemi 2004; Banihashemi and Ghaderi 2006).

Table 1. *Phytophthora* isolates used in phylogenetic analyses in this study (Sequences were generated in this study are in bold letters).

Species name	**strain**	**GenBank accession no.**	
		βtub	***tef1-α***
Phytophthora acerina	61H1	KX250713	KX250714
P. bisheria	P10117	EU080742	EU080743
P. botryosa	46C2	KX250531	KX250532
P. capsici	22F4	KX250636	KX250637
P. capensis	62C1	KX250727	KX250728
P. citricola	33H8	KX250748	GQ247662
P. citrophthora	03E5	KX250545	KX250546
P. colocasiae	35D3	KX250566	KX250567
P. elongata	33J4	KX250888	KX250889
P. frigida	47G7	KX250909	KX250910
P. glovera	62B4	KX250650	KX250651
P. multivesiculata	30D4	KX250923	KX250924
P. multivora	55C5	KX250776	KX250777
P. infestans	27A8	KX250475	KX250476
P.inflate	P10341	EU080385	EU080386
P. meadii	61J9	KX250594	KX250595
P. mengei	42B2	KX250657	GQ247669
P. mexicana	45G4	KX250671	KX250672
P. pini	22F1	KX250804	GQ247665
P. plurivora	61H1	KX250713	KX250714
P. occultans	65B9	KX250601	KX250602
P. occultans	**Sans-Po1**	**OQ267674[a]**	**OQ267668**
P. occultans	**Sans-Po2**	**OQ267675**	**OQ267669**
P. occultans	**Sans-Po3**	**OQ267676**	**OQ267670**
P. occultans	**Sans-Po4**	**OQ267677**	**OQ267671**
P. occultans	**Sans-Po5**	**OQ267678**	**OQ267672**
P. occultans	**Sans-Po6**	**OQ267679**	**OQ267673**
P. siskiyouensis	41B7	KX250678	KX250679
P. tropcalis	22H5	KX250692	KX250693

The plants were inoculated with 10cc of inoculum at the base of sansevieria and covered with soil. Control plants were treated only with vermiculite containing hemp seed extract. Then pots were flooded with the drainage hole closed with melted paraffin. The next day, the drainage holes were opened and water from each pot was collected, filtered through cheesecloth and baited with citrus leaf disks. After 48 h, the leaf disks blotted dry with sterile paper towels, plated on CMA-PARPH medium at 25 °C. With the emergence of the fungus colonies on CMA-PARPH medium, boiled hemp seeds were placed on colonies for 12-24

hours, then transferred into sterile distilled water in plastic Petri dishes and incubated at 25°C under continuous fluorescent illumination for 2-3 days. In fact, sporangia formation around the colonized hemp seeds gave a positive indication of *Phytophtora* activity (Banihashemi et al. 1992; Kannwischer and Mitchell, 1978). The inoculated sansevieria plants were maintained at 20-32°C for one month under natural daylight conditions in the greenhouse. The causal agents were re-isolate from symptomatic plants to fulfill the Koch's postulates.

RESULTS AND DISCUSSION

Up until now, species of *Phytophthora* reported to infect sansevieria root, and crown worldwide were *P. nicotiana* from Clade 1 of *Phytophthora* phylogeny (Patel et al. 2016). In Iran, there are no reports of root rot on sansevieria. Thus, the main goal of this study was to identify *Phytophthora* species causing sansevieria root rot in the Isfahan Province of Iran.

The symptoms of the disease were wilting, necrosis of the leaves, stem base and crown rots, and eventually plant mortality (Fig. 1).

In total, six *Phytophthora* isolates were recovered from sansevieria; four isolates obtained from infected roots tissue, and two from the root zone soil of infected plants using baiting method while no isolates were recovered from the root zone soil of healthy plants (Table 1). Based on morphological and molecular characteristics, isolates belonged to *Phytophthora occultans* that were found on sansevieria in Isfahan Province, Iran.

Phytophthora occultans isolates formed semi-papillate sporangia abundantly on liquid medium, sometimes bipapillate sporangia, ovoid to obpyriform, caducous and non-caducous, 25.2–32.7 × 39.8–54.5 µm.

All *Phytophthora occultans* isolates were homothallic and were able to produce oospores abundantly in a single HSA culture. Oospores were mostly aplerotic, spherical, 23.9–25.8 µm in diameter. Oogonia were globose, smooth, 26.5–28 µm in diameter with paragynous antheridia and seldom amphigynous. Chlamydospores were not produced by any of the isolates on different (media CMA, MEA, PCA, PDA, HAS and V8A). Hyphae were hyaline and hyphal swellings were absent (Fig. 2a-g). The minimum, optimum, and maximum growth temperatures for *Phytophthora occultans* on CMA medium were10, 25, and 30 °C, respectively.

Fig. 1. Symptoms of root and crown rots caused by *Phytophthora occultans* on sansevieria in greenhouses of Isfahan Province, Iran.

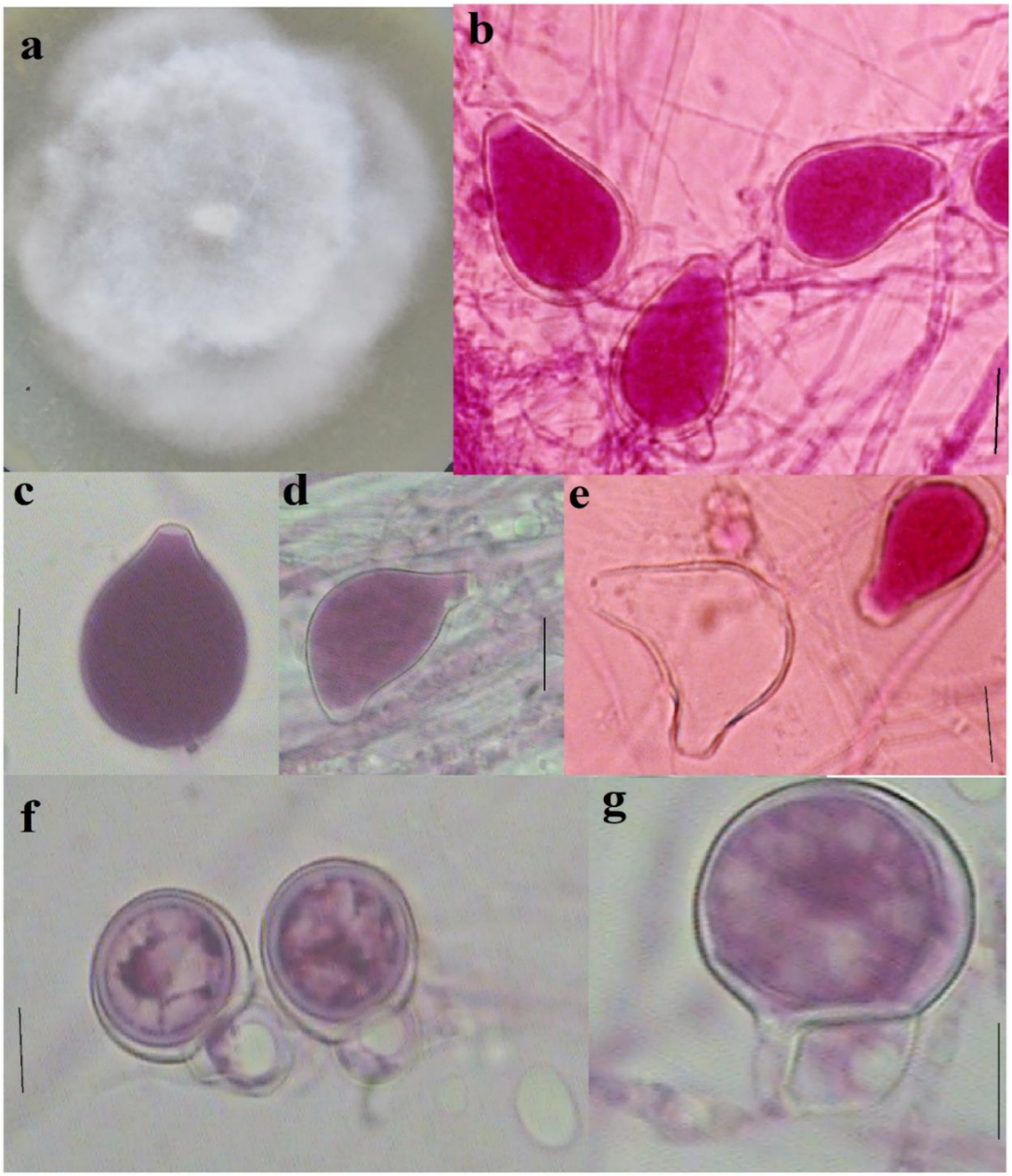

Fig. 2. Morphological structures of *Phytophthora occultans*. (a) Colony morphology on CMA at 25 °C. (bc) Semi-papillate sporangia. (d) Bipapillate sporangia. (e) Bipapillate sporangium after release of zoospores. (f) Oogonia with paragynous antheridia and aplerotic oospores. (g) Oogonia with paragynous antheridium and an aborted oospore. Scale bar = 20 µm.

PCR amplification and sequencing of *tef1-α* and *βtub* genes were successful for six *Phytophthora* isolates. The aligned data sets of Clade 2 for *βtub* and *tef1-α* consisted of 1137 and 1016 characters, respectively. The aligned multigene data set of Clade 2 taxa contained 2017 characters. The obtained sequences of *Phytophthora* were submitted to GenBank under the following GenBank Accession No.: OQ267668 to OQ267673 for tef1-α gene and OQ267674 to OQ267679 for βtub gene (Table 1). Phylogenetic analyses based on *tef1-α* and *βtub* sequences revealed the position of six recovered in Clade 2 of *Phytophthora* spp., which formed a monophyletic group with isolate 65B9 of *P. occultans* (Yang *et al.* 2017) in a high bootstrap value (Fig. 3).

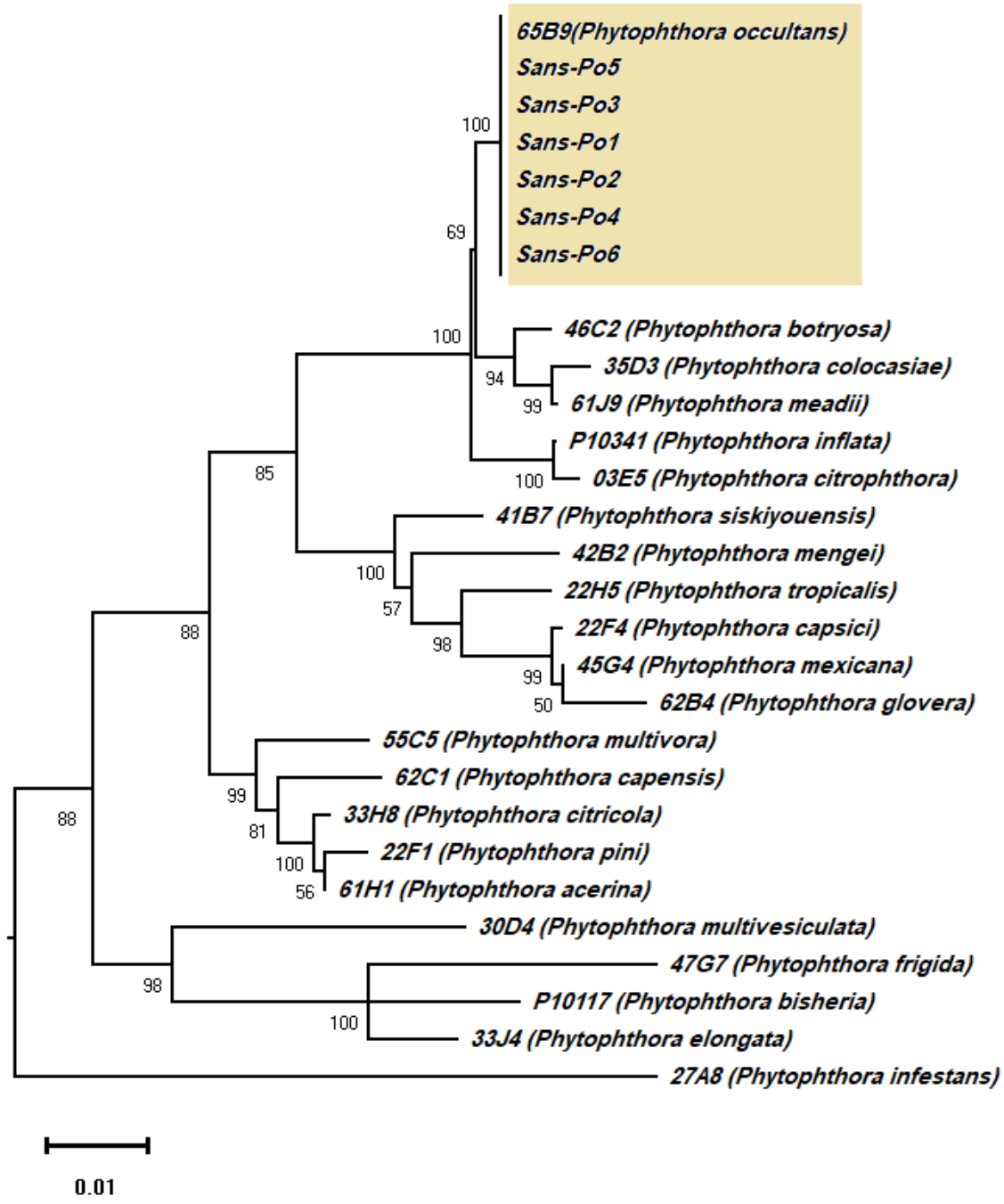

Fig. 3. Maximum Likelihood phylogenetic tree inferred from *βtub* and *tef1-α* genes of Clade 2 of *Phytophthora* species and isolates recovered from sansevieria from Isfahan Province, Iran. Bootstrap values (in %) have been shown under the branches. *Phytophthora infestans* is used as an outgroup.

In this study, Isolates Sans-Po1 to Sans-Po6 of *Phytophthora occultans* were pathogenic on sansevieria plants and caused root and crown rots after one month of inoculation (Fig. 4). In general, infected plants showed wilting, and root and crown rots. No symptoms were observed in noninoculated control plants. The causal agents were reisolated from infected plants but not from controls. Koch's postulates were completed and confirmed that *Phytophthora occultans* isolates were responsible for sansevieria root and crown rots.

The overall topologies of our phylogenetic trees of Clades 2 was consistent with Yang et al. (2017). Phylogenetic analysis of *tef1-α* and *βtub* genes clustered *Phytophthora* spp. isolates from sansevieria in Isfahan province within Clade 2. This clade is one of the main clades in the *Phytophthora* spp. phylogeny and comprises significant pathogenic species such as *P. capsici, P. citricola, P. plurivora, P. multivora, P. tropicalis,* and *P. citrophthora* (Kroon et al. 2012).

In general, morphological and molecular characterization is needed to identify *Phytophthora* species (Yang et al. 2017; Puglisi et al. 2017). The ITS-rDNA region may, in some cases, not be appropriate to differentiate *Phytophthora* species (Schena and Cooke 2006), consequently, we selected two *tef1-α* and *βtub* genes that supported a precise and reliable identification.

Morphological and phylogenetic analyses of the DNA sequence data of *tef1-α* and *βtub* let us identify *Phytophthora occultans* associated with root and crown rot of sansevieria in greenhouses of Isfahan Province. To our knowledge, this is the first report of this disease on sansevieria in Iran. In addition, after an intense survey, there were no reports of *P. occultans* causing root rot in sansevieria worldwide.

Phytophthora occultans Man in 't Veld & K. Rosend was first isolated from rotted roots of *Buxus sempervirens* L. in 1998 from the Netherlands then were ignored for more than 10 years, and re-emerged in 2010 (Man In't Veld et al. 2015). *Buxus sempervirens*, the major host of *P. occultans*, was famous in Europe in Roman times and it is probable that movement of *B. sempervirens* was responsible for the introduction of *P. occultans*. A possible candidate for the introduction of *P. occultans* is *Acer palmatum*, presently imported from China and Japan in large quantities. After its introduction, the pathogen could distribute through horticultural centers. Until now, this species has been isolated from four genera including *Taxus* sp., *B. sempervirens*, *Choisya ternate* Kunth[and *Acer palmatum* Thunb (Man In't Veld et al. 2015). *Phytophthora occultans* caused considerable losses of approximately $5,000,000 in the Netherlands (Man In't Veld et al. 2015). Man In't Veld et al. (2015) explained that this species possibly would be stowaways on host plants with few or no disease symptoms or in the soil in which imported plants frequently were transported.

The presence of this pathogen in the greenhouses of Isfahan Province is worrying because, besides sansevieria, other ornamental plants are also growing in these greenhouses, which may be infected with this pathogen. This newly introduced pathogen has greatly affected sansevieria and other ornamental plants producers in Iran. This species is easily carried by plant materials worldwide. The import of plant materials from infested green houses and nurseries must be prevented. This aspect is significant from the epidemiological point of view for plant diseases, considering that long-distance distribution of most pathogens occur mostly through the transit of contaminated plant material, and human activities are recognized as a major mode of introduction of fungal plant pathogens (Brasier, 2008). This pathogen may pose a threat to greenhouse crops. Intensive sampling of greenhouses in many provinces of Iran is needed to recognize which other *Phytophthora* species presently reside in the greenhouses.

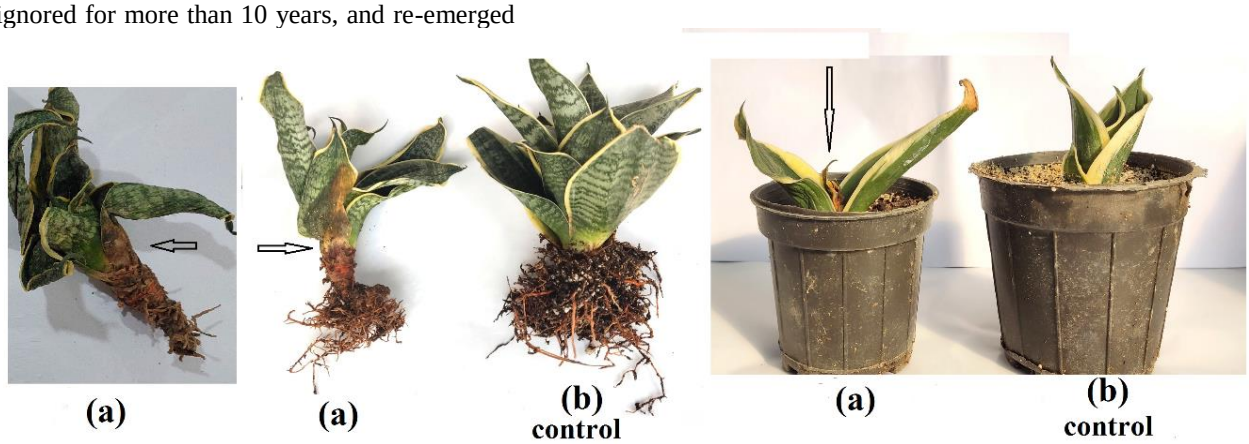

Fig 4. Sansevieria plants showing symptoms of root and crown rots after one month of inoculation with *Phytophthora occultans* : (a) infected plants, (b) control.

The information from this research is useful to greenhouses by providing data about a pathogen threatening ornamental plants. We suggest purchasing *Phytophthora*-free transplants for planting to avoid transplant-borne diseases, by examining the transplants for the presence of any *Phytophthora* species and limiting periods of soil saturation in greenhouses in order to reduce the dispersal of zoospores to healthy plants because we obtained no isolates from the root zone soil of healthy plants using baiting method.

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گزارش جدید از *Phytophthora occultans*، عامل پوسیدگی ریشه سانسوریا

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چکیده: در تابستان ۱۴۰۱، گیاهان سانسوریا با علائم پوسیدگی ریشه در گلخانه‌های استان اصفهان مشاهده گردید. بافت‌های آلوده روی محیط‌کشت CMA-PARPH کشت شد. جدایه‌های اُمیستی به دست آمده به روش تک زئوسپور روی محیط‌کشت WA خالص‌سازی گردید. از روش طعمه‌گذاری با استفاده از برگ مرکبات برای جداسازی جدایه‌های فیتوفتورا از خاک اطراف ریشه استفاده گردید. همه جدایه-های اُمیستی براساس ویژگی‌های ریخت‌شناختی و مولکولی (ژن‌های *βtub* و *tef1-α*) شناسایی شدند. مطابق واکاوی‌های فیلوژنتیکی ریخت-شناختی، شش جدایه، *Phytophthora occultans* تشخیص داده شد. این نخستین گزارش از *Phytophthora occultans*/ست که باعث پوسیدگی ریشه‌ی سانسوریا از ایران و احتمالاً دنیا می‌باشد.

کلمات کلیدی: اُمیکوتا، ایران، اصفهان، سانسوریا، *Dracaena trifasciata*، *β*-tubulin، *tef1-α*

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