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Identification and molecular characterization of *Phyllosticta yuccae* isolates causing Yucca leaf spot in Iran

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Abstract: During 2017-2018, the leaf spot disease was observed in mound-lily Yucca (*Yucca gloriosa*) and Spineless Yucca (*Y. elephantipes*) in Tehran, Fars and Bushehr provinces of Iran. In order to identify the causal agents of the disease, infected tissues were collected and transferred to the laboratory and 27 fungal isolates were isolated. Fungal isolates were identified based on morphological characteristics and molecular data of the internal transcribed spacer (ITS) region and parts of the actin (*act*) and the translation elongation factor 1-alpha (*tef1*) genes. According to the morphological and phylogenetic analysis, the isolates were identified as *Phyllosticta yuccae*. The pathogenicity test was performed on healthy and attached leaves of *Y. gloriosa* and *Y. elephantipes* plants. Inoculated plants showed leaf spot symptoms in seven days' post inoculation, while control plants remained symptomless. To complete the Koch's postulate, *P. yuccae* was re-isolated from inoculated plants. The results of this study revealed that the causal agent of leaf spot disease of Yucca plants was *P. yuccae*.

Keywords: Pathogenicity, ornamental plants, ITS, *act*, *tef1*

INTRODUCTION

Yucca sp. (family Asparagaceae) is widely grown as an ornamental plant (Chase et al. 2009). Yucca plants are native to the hot and dry regions of the Americas and Caribbean and tolerate a wide range of conditions (Irish & Irish 2000, Dorling 2008). Many species of Yucca plants such as *Yucca elephantipes*, are commonly grown as houseplants, while some of them including *Y. filamentosa*, *Y. flaccida*, and *Y. gloriosa* are commonly grown outside in gardens (Irish & Irish 2000). This ornamental plant, which is cultivated as a perennial shrub and tree, provides a dramatic accent to landscape design (Chase et al. 2009).

Ornamental plants, like the other plant species, are affected by various fungal species causing leaf spot diseases. According to the surveys in Iran, different species of *Alternaria*, *Ascochyta*, *Cercospora*, *Cladosporium*, *Colletotrichum*, *Coniothyrium*, *Ectophoma*, *Fusarium*, *Glomerella*, *Graphiola*, *Pestalotiopsis*, *Phoma*, *Phyllosticta*, and *Septoria* have been reported as the causal agents of leaf spot on different ornamental plants (Azimi-Motem & Osipyan 2009, Ershad 2009, Bagherabadi et al. 2018, Hosseinnia & Mohammadi 2018, Larki et al. 2018, Bakhshi et al. 2019, Bakhshi & Zare 2020). Since the beauty of the ornamental plants is the major factor in their cultivation, any pathogenic problem could be serious from the cultivator's point of view. To that end, infected plants are never accepted in flower gardens and they are not profitable in commercial cultivations at all (Tilford 1932). Identification of leaf spot agents on ornamental plants can be helpful in plant disease management and lead to prevent their spread and progress of the disease. Therefore, the aim of this study was to identify and characterize the *Phyllosticta* sp. isolated from Yucca plants in Iran, using morphological and molecular characteristics and test them for pathogenicity.

MATERIALS AND METHODS

Fungal isolation and morphological characterization

During 2017-2018, mound-lily Yucca (*Yucca gloriosa*) and Spineless Yucca (*Y. elephantipes*) plants showing typical leaf spot symptoms were collected from different areas in Fars, Bushehr, and Tehran provinces of Iran (Table 1). Small and excised portions of leaves with characteristic lesions were sterilized in 1% sodium hypochlorite, washed twice with sterile distilled water, dried using filter paper, and placed onto potato dextrose agar medium (PDA).

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Table 1. Details and GenBank accession numbers of *Phyllosticta* strains included in this study.

Species	Culture no.✦	Host	Country	GenBank no.		
				ACT	ITS	*tef1*
Phyllosticta capitalensis	CBS 128856^T	*Stanhopea* sp.	Brazil	JF343647	JF261465	JF261507
	CPC 27061	*Citrus limon*	Italy	KY855643	KY855588	KY855917
	CBS 226.77	*Paphiopedilum callosum*	Germany	FJ538452	FJ538336	FJ538394
	CBS 101228	*Nephelium lappaceum*	Hawaii	FJ538435	FJ538319	FJ538377
	CPC 14609	*Zyzygium* sp.	Madagascar	KF289264	KF206184	KF289175
	CPC 20263	Magnoliaceae	Thailand	KC342538	KC291341	KC342561
	GZAAS6.1202	*Musa* sp.	China	KM816624	KF955291	KM816636
	GZAAS6.1242	*Musa* sp.	China	KM816625	KF955292	KM816637
P. paracapitalensis	CPC 26517^T	*Citrus floridana*	Italy	KY855677	KY855622	KY855951
	CPC 28129	*Citrus limon*	Spain	KY855689	KY855634	KY855963
	CBS 173.77	*Citrus aurantiifolia*	New Zealand	KF289244	KF206179	FJ538393
P. psidii	CBS 100250	*Psidium guajava*	Brazil	FJ538467	FJ538351	FJ538409
Guignardia mangiferae	IMI260.576	*Mangifera indica*	India	JF343641	JF261459	JF261501
	CPC 20260	Arecaceae	Thailand	KF289294	KF206193	KF289187
P. citricarpa	CPC 28104	*Citrus sinensis*	Portugal	KY855673	KY855618	KY855947
	CBS 127454^T	*Citrus limon*	Australia	JF343667	JF343583	JF343604
P. aloeicola	CPC 21021	*Aloe ferox*	South Africa	KF289312	KF154281	KF289194
	CPC 21020^T	*Aloe ferox*	South Africa	KF289311	KF154280	KF289193
P. yuccae	CBS 117136	*Yucca elephantipes*	New Zealand	JN692517	JN692541	KF766436
	YM1	***Yucca gloriosa***	**Iran- (Fars province)**	**MT358806**	**MT241827**	**MT358817**
	Ysh2	***Yucca gloriosa***	**Iran- (Fars province)**	**MT358807**	**MT241828**	**MT358818**
	YB1	***Yucca elephantipes***	**Iran- (Bushehr province)**	**MT358808**	**MT241829**	**MT358819**
	YT3	***Yucca elephantipes***	**Iran-(Tehran province)**	**MT358809**	**MT241830**	**MT358820**
P. rubella	CBS 111635 ^{T}	*Acer rubrum*	USA	KF289233	KF206171	KF289198
P. citriasiana	CBS 120486 ^{T}	*Citrus maxima*	Thailand	FJ538476	FJ538360	FJ538418
P. cordylinophila	CPC 20277	*Cordyline fruticosa*	Thailand	KF289301	KF170288	KF289171
	CPC 20261^T	*Cordyline fruticosa*	Thailand	KF289295	KF170287	KF289172
P. paracitricarpa	CPC 27169^T	*Citrus limon*	Greece	KY855690	KY855635	KY855964
	CPC 27170	*Citrus limon*	Greece	KY855691	KY855636	KY855965
Diplodia seriata	CMW 8232	*Conifers* sp.	South Africa	AY972111	AY972105	DQ280419

✦T: Type strain Strains isolated in this study are in boldface.

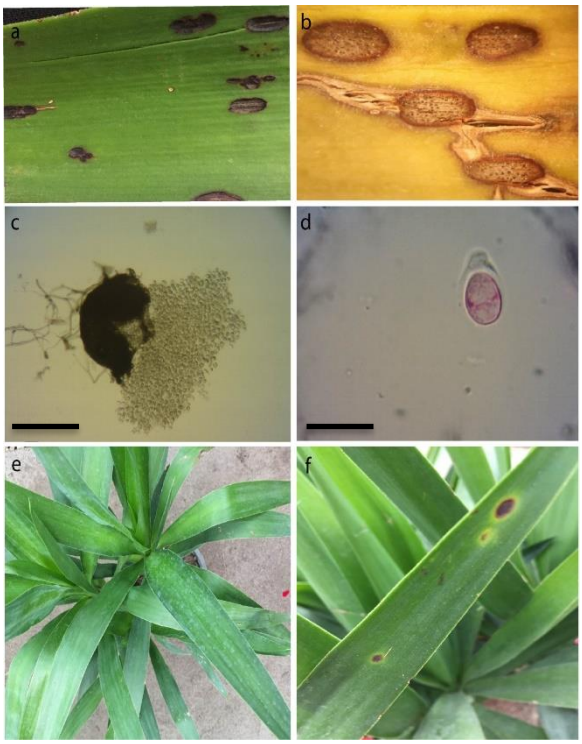

Fig. 1. Leaf spot symptoms on leaves of wild Yucca plants (a-b); *Phyllosticta* pycnidia (c); conidia (d); non-inoculated plant (e); leaf spot symptoms on leaves two weeks after inoculation (f). — Scale bars = 20 µm.

The fungal colonies grown from infected tissues were purified using single spore technique (Sinclair & Dhingra 1995). Cultural characteristics and morphological features of the isolates were determined on PDA, 2% malt extract agar (MEA; 20 g/L malt extract, 16 g/L agar), and oatmeal agar (OA; 60 g/L oatmeal, 16 g/L agar) media (Bissett 1986, Wikee et al. 2013).

DNA extraction, amplification, and phylogenetic analysis

BLAST search (which is available at https://blast.ncbi.nlm.nih.gov/) was conducted to compare newly obtained sequences against NCBI database. All sequences used in this study are listed in Table 1. Sequences were aligned using CLUSTAL W program and concatenated following alphabetic order of the genes, ending in a sequence of 951 base pairs: nucleotides 1 to 223 for *act*, 224 to 718 for ITS, and 719 to 951 for *tef1*.

The best evolutionary model was determined using the Modeltest option from MEGA 6.06. Phylogenetic trees were constructed using the maximum likelihood algorithm (Tamura et al. 2013). *Diplodia seriata* (CMW25477) was chosen as an outgroup taxon. The bootstrap value was adjusted to 1,000 replications. The final tree was illustrated with infix pdf editor (https://www.iceni.com/infix.htm).

Pathogenicity test

The pathogenicity test was performed using the protocol described by Wikee et al. (2013) with three treatments as wounded leaves, unwounded leaves, and control. Before inoculation, attached and healthy leaves of two plant species (*Y. gloriosa* and *Y. elephantipes*) were surface sterilized using 70 % ethanol and washed three times with sterile, distilled

water. The leaves were then dried with sterile tissue paper. Mycelial plug inocula (0.7 mm) were obtained from the edges of 15-day-old cultures and transferred to the wounded and unwounded leaves. For control treatments, leaves were inoculated with PDA plugs. All inoculated plants were then incubated in a moist chamber. Two weeks after inoculation, the fungal isolates on the inoculated leaves with leaf spot symptoms were re-isolated and cultured on PDA. The morphological characteristics of the isolates were compared with those of the original isolates from the leaf spots to confirm Koch's postulates.

RESULTS AND DISCUSSION

The disease symptoms including dark red-brown, irregular and circle necrotic spots, were observed on the Yucca leaves (Fig. 1 a). Dark brown spots commonly have subglobos pycnidia on the upper side of the leaf (Fig. 1 b). A total of 27 strains of *Phyllosticta* associated with Yucca leaf spots were collected from three different geographical locations in Iran (Table 1). Fifteen strains isolated from infected *Y. gloriosa* and 12 strains isolated from *Y. elephantipes* were morphologically identical. In all strains, conidiogenesis was blastic, and the conidiogenous cells were cylindrical, hyaline, and 5.7 to 9.5 × 2.8 to 6 µm. Conidia were one-celled, aseptate, hyaline, smooth-walled, coarsely granular, broadly ellipsoidal to subglobose or obovate, usually broadly rounded at both ends, and 7.5 to 14.6 × 6 to 9.5 µm. Conidia were also surrounded by a slime layer about 1 µm wide, usually with a hyaline, flexuous, narrowly conoidal or cylindrical, mucilaginous apical appendage that was 4 to 12 µm long (Fig. 1 c, d). These characteristics matched well with the description of *Phyllosticta yuccae* (Bissett 1986). The representative isolate (Ysh2) was deposited in the Culture Collection of the Iranian Research Institute of Plant Protection, Tehran, Iran (IRAN 4218C).

The morphological identification of the fungus was confirmed with molecular analysis and phylogenetic approach. The results of molecular identification based on three different loci and blasted against the available sequences in GenBank showed that the strains belong to *P. yuccae*.

Phylogenetic tree constructed with concatenated sequences of the *act*, ITS, and *tef1* showed that four strains isolated in Iran were clustered with *P. yuccae* strain (CBS 117136) with 2, 4, and 4 different nucleotides in the sequences of *act* (site: 160 and 182), ITS (Site: 252, 371, 394, and 456), and *tef1* (Site: 739, 807, 813, and 818), respectively (Fig. 2). Iranian strains were grouped close to *P. rubella*, but separated from *P. rubella* with 5, 5, and 8 different nucleotides in the sequences of *act* (Site: 69, 76, 88, 96, and 151), ITS (Site: 247, 319, 364, 394, and 578), and *tef1* (771, 772, 779, 812, 814, 856, 869, and 908), respectively.

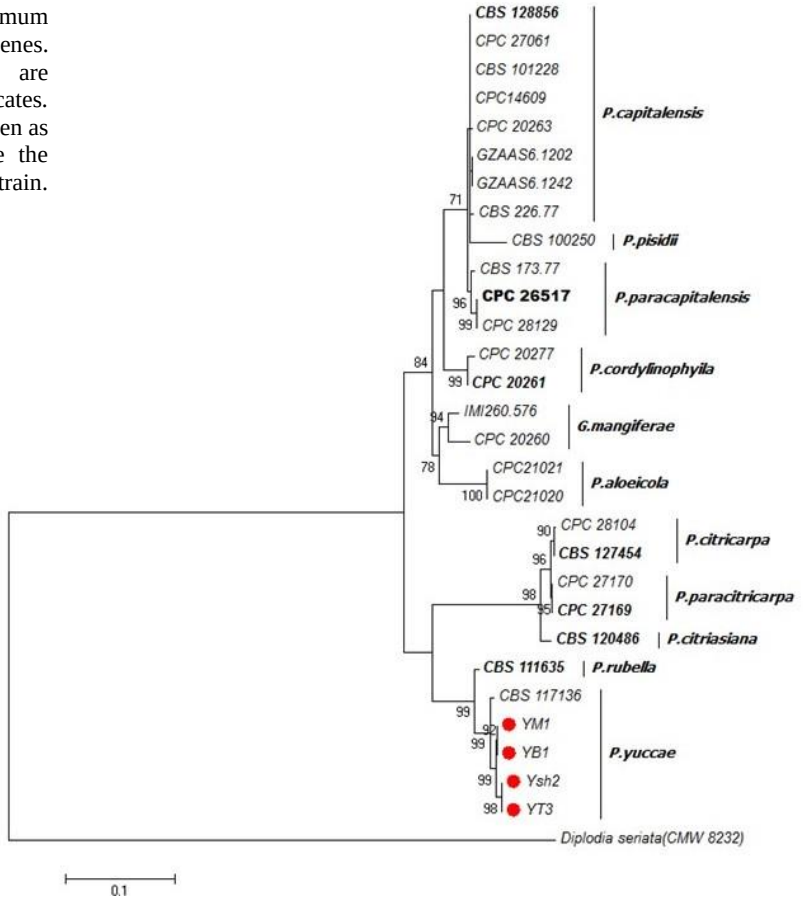

Fig. 2. Concatenated maximum likelihood tree of *act*, ITS and *tef1*genes. Bootstrap values higher than 70% are shown as percentages of 1000 replicates. *Diplodia seriata* CMW 8232 was chosen as outgroup taxon. Red circles indicate the strains isolated in this study. T: Type strain.

The results of the pathogenicity test demonstrated that *P. yuccae* is the causal agent of leaf spots on *Y. elephantipes* and *Y. gloriosa*. Symptoms like dark brown and necrotic elliptical spots were observed on inoculated leaves in seven days after inoculation. The results showed symptoms similar to those occurred in naturally infected plants (Fig. 1 e, f). Species in the genus *Phyllosticta* have been reported as plant pathogens, endophytes, and saprobes (Baayen et al. 2002, van der Aa & Vanev 2002, Okane et al. 2003, Glienke et al. 2011). A wide range of economically important crops and ornamental plants are affected by many species of *Phyllosticta*, which lead to leaf spot symptoms and fruit diseases. For example, *P. yuccae* has previously reported as a causal agent of leaf spot disease on *Y. filamentosa* in Brazil (Glienke-Blanco et al. 2002, Silva & Pereira 2007, Silva et al. 2013).

Currently, more than 20 species of *Phyllosticta*, including *P. hedericola*, *P. theae*, and *P. yuccae* have been reported affecting different plant species such as *Camellia japonica*, *Hedera helix*, *Magnolia grandiflora*, *Rosa* sp., *Schefflera* sp., *Syringa reticulate*, and *Y. elephantipes* from Iran (Fatehi & Mirabolfathy 1994, Ershad 2009, Darsaraei et al. 2016, Esmaeilzadeh et al. 2020) It is for the first time that molecular identification (based on three loci) and the pathogenicity test of *P. yuccae* on *Y. gloriosa* have been studied in Iran.

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شناسایی مولکولی و بیماری‌زایی جدایه‌های *Pyllosticta yuccae*، عامل لکه‌برگی یوکا در ایران

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چکیده: در طی بازدیدهایی که در سال‌های ۱۳۹۶ و ۱۳۹۷ از استان‌های فارس، تهران و بوشهر انجام شد، نشانه‌های لکه‌برگی در گیاهان یوکا زنگوله‌ای (*Yucca gloriosa*) و یوکا فیلی (*Y. elephantipes*) مشاهده شد. به‌منظور بررسی عامل بیماری، نمونه‌های آلوده پس از جمع‌آوری به آزمایشگاه منتقل شدند و ۲۷ جدایه قارچی از گیاهان آلوده جداسازی شد. شناسایی جدایه‌ها براساس ویژگی‌های ریخت‌شناسی و توالی‌یابی ناحیه ITS و بخشی از ژن‌های *act* و*tef1* انجام شد. براساس بررسی‌های ریخت‌شناسی و واکاوی‌های تبارشناسی مبتنی بر ترکیب توالی‌های مورد بررسی، این جدایه‌ها به عنوان گونه *Phyllosticta yuccae* شناسایی شدند. آزمون بیماری‌زایی بر روی گیاهان سالم یوکا زنگوله‌ای و یوکا فیلی انجام شد. گیاهان مایه‌زنی شده بعد از یک هفته، نشانه‌های لکه‌برگی را نشان دادند، در حالی که گیاهان شاهد فاقد هر گونه نشانه بودند. به منظور تایید اصول کخ، *P. yuccae* به طور مجدد از برگ‌های دارای علائم جداسازی شد. بر اساس نتایج بررسی‌های انجام شده، قارچ عامل لکه برگی در گیاهان زینتی یوکا در این پژوهش *P. yuccae* می‌باشد.

کلمات کلیدی: بیماری‌زایی، گیاهان زینتی، ITS، *act*، *tef1*

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