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Research Article

Fusarium acuminatum: A new pathogen causing yellows and wilt on Dragon's head *Lallemantia iberica* in Iran

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Abstract: Dragon's head *Lallemantia iberica*, currently is grown as an oilseed crop in dry areas of Iran. In 2019, symptoms including seedling damping-off, yellows, and wilt were observed on the plants in a commercial field in Maragheh province, Iran. Based on the morphological and molecular characters, the fungus isolated from symptomatic plants was identified as *Fusarium acuminatum*. The pathogenicity of the fungus was confirmed through inoculation of the host plant. Subsequently, Koch's postulates were fulfilled by re-isolation of the same fungus from the inoculated symptomatic plants. This is the first report of Fusarium wilt disease occurring in Dragon's head in Iran and worldwide.

Keywords: Translation elongation factor 1-a, wilt and yellows, damping off, oilseeds, fungal etiology, Maragheh

Introduction

Oilseeds are ranked as the fourth most important crop in the world (FAO, 2015). They are considered a great energy source in the form of oils and fats (McKevith, 2005) and supply 20-30% of the calorie demand of an average, healthy adult (Attia *et al*., 2021). According to FAO (FAO, 2022a; FAO, 2022b), although the world production of oilseed crops, including linseed, okra, rapeseed, safflower, cottonseed, sesame, sunflower, groundnut, soybeans, and mustard has increased by 29.8% and reached 597 million tons during the last decade, the demand is still dramatically high. Their price index increased by 12% in 2022.

Dragon's head *Lallemantia iberica* (M. Bieb.) Fisch. & C.A. Mey, in Persian "Balangu-Shahri", is a highly valued annual plant that belongs to the family of Lamiaceae. Its seeds and aerial parts are regarded as a valuable source of various natural compounds such as oils, fatty acids, secondary metabolites, lipids, sterols, and volatile oils (Samadi *et al*., 2007; Nikitina *et al.*, 2008; Nori-Shargh *et al.*, 2009; Asghari *et al.*, 2017). Although it is used for cultivation as a medicinal or ornamental plant in the Middle East or Eastern Europe (Ozdemir *et al*., 2014; Komartin *et al.*, 2021; Naghibi and Motamed, 2005; Overeem *et al.*, 1999; Sadeghi-Varkani *et al.*, 2018; Zlatanov *et al.*, 2012), recently it is cultivated also in dry areas of Iran as oilseed crop (Rostami Ahmadvandi and Faghihi, 2021) due to its significant capability to tolerate environmental stresses such as drought and salinity (Omidi *et al.*, 2018; Abdolahi and

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Maleki Farahani, 2019). There are no data on the pests or diseases of this crop in literature and the objective of this study was the identification of pathological agent of a disease observed in this crop in Iran.

Materials and Methods

Plant sample collection, isolation, and morphological identification

A typical yellowing and wilt along with seedling damping-off symptoms were observed in a field of the crop in Maragheh (37º 16' 15" N 46º 25' 33" E), Iran, in the year 2018-19 growing season. An upward expansion of the symptom was observed on the infected plants, leading to broken stems, collapse, drooping, and finally, death. The disease incidence in the region reached 40% (Fig. 1). Twenty infected plant samples were taken from the field and transferred to the lab. Isolation of the pathogen from symptomatic tissue is an excellent technique to obtain pathogenic agents from an infected plant (Gareth Jones, 1987); so, small segments of the symptomatic stems were surface sterilized with 0.5% NaOCl, rinsed twice in the distilled water, plated on the quarter-strength potato dextrose agar (QPDA) (Salas *et al.*, 1999), and incubated at 25ºC in 12 h darkness/ 12 h light photoperiod with supplemental near UV light. A typical Fusarium-like colony appeared on the cultures by seven days post inoculation (dpi) from all samples. Purification and sub-culturing of the isolated fungi were conducted on QPDA amended with 1mg/mL chloramphenicol (Fulton *et al.*, 2021). The fungal colonies were subjected to morphological studies using the classification keys of Nelson *et al.* (1983) and Summerell *et al.* (2011) on Carnation Leaf Agar (CLA) media culture (Fisher *et al.*, 1982). Out of thirty isolated colonies derived from infected plants, one morphologically representative isolate was subjected to further studies.

Figure 1 Field symptoms and cultural characteristics of *Fusarium acuminatum* infected Dragon's head *Lallemantia iberica*. (A) Crop loss in the field, (B) Mature plants showing leaf rolling, yellowing and wilt symptoms, (C) Damping-off in the seedlings sampled from the field.

Molecular identification

A pure culture was multiplied in PDB (200 g potato, 20 g dextrose, 1 L water) on the shaker at 28-30ºC /160 rpm for 3-5 days. The liquid was filtered through a 4-layer cheesecloth, 20-40 mg of the mycelium was harvested using a small spatula, then lyophilized and ground in a clay mortar. Subsequently, the genomic DNA (gDNA) of the mycelium was extracted according to the method proposed by Dellaporta *et al.* (1983). The translation elongation factor-1α (*tef1-α*) region was amplified using the primer pairs EF1/EF2 (O'Donnell *et al.*, 1998). The primer was amplified in a total volume of 40 µL, containing 16 µL Master mix PCR (Ampliqon ®, Denmark, ID 5200300-1250, Batch 21H2601), 1.5 mM $MgCl_2$, 50 ng gDNA, 8 µL sterilized double deionized water and 4µL of each primers (10 µM). The reaction was performed on a Senso Quest ® (Germany) PCR system. PCR amplification conditions

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were according to Carbone and Kohn (1999) with minor modifications: initial denaturation at 95 ºC for 8 min followed by 35 cycles of denaturation at temperature of 95 ºC for 15s, primer annealing at 55 ºC for 20s, primer extension at 72 ºC for 60s, and a final extension step at 72 ºC for 5 min. Amplification products were separated in standard 1–1.5% agarose gel electrophoresis for 120 min at 90 V in 1X TBE buffer (90mM TRIS base, 90 mM boric acid and 2 mM Na_2EDTA). To visualize the amplified DNA, gels were stained with DNA self-stain (3µL/100 mL), then photographed by trans illumination using Biocom Direct ® (England) gel-documentation system. A distinct DNA product was obtained at the suspected size of 700 bp. The produced gel fragments of the primer in both directions were sequenced in Microsynth AG Ca. ®, Switzerland. The obtained sequence was edited using Bioedit (Hall, 1999) software and deposited in GeneBank as MZ851155**.** The amplified sequence was compared with other sequences found in the National Center for Biotechnological Information database (NCBI) (https://www.ncbi.nlm.nih.gov) using the Standard Nucleotide Basic Local Alignment Search Tool (BLASTn).

Phylogenetic analysis

The taxonomic relationship of this fungus with other *Fusarium* species was investigated using MEGA11 *(*Tamura *et al.*, 2021) software by constructing a phylogenetic tree. Twenty-six sequences of the same gene belonging to the different *Fusarium* species were taken from GenBank (Table 1) using BLASTn and were aligned with the studied isolate using Clustal-W (Thompson *et al.*, 1994) method. The lowest Bayesian Information Criterion (BIC) was achieved via the application of Hasegawa-Kishino-Yano using a discrete Gama distribution substitution model. The Maximum Likelihood (ML) statistical method was performed and tested with the Bootstrap method at a rate of 1000 replications. Gamma distribution was applied to regulate site rates (Jia *et al.*, 2014). Sites with missing data were moderately removed from the model using the Partial Dilation option.

Pathogenicity test

The standard root-dip method (Martyn and Mclaughlin, 1983; Freeman and Rodriguez, 1993) with minor modifications was applied for inoculation of the plants with fungus. Two hundred fifty seeds of an Iranian landrace of Dragon's head were surface-sterilized in NaOCl 0.5% for 10 min, rinsed twice in sterile distilled water, put in Petri dishes containing wet filter paper in the bottom, and kept at 17 ºC for one week to be germinated. Subsequently, the germinated seeds were transferred into 5 × 7 × 8 cm pots containing sterile perlite and kept at 25 ºC with a 12 h photoperiod of 300 µE m^{-2} S^{-1}. Twenty germinated seeds were sown in each pot, and 10 pots were prepared (200 plants). Spores of the fungal isolate were multiplied on PDB at 28-30 ºC for 3-5 days on a shaker at 160rpm. The medium was filtered through a four-layer sterilized cheesecloth, centrifuged at 750 rpm for 14 min, and conidia concentration was adjusted at 5×10^5 spores per mL. All plants were inoculated at the 2-4 leaf stage by cutting the tip of the roots and immersing in the spore suspension for 1 min, then replanting in the pots. Control plants were treated by the same method and dipped in sterile water. After inoculation, the plants were irrigated with full Hogland's solution ($Ca(NO_3)_2$:$4H_2O$: KH_2PO_4: KNO_3: $MgSO_4$: (5:1:5:2 nM): $7H_2O$: FeEDTA (5µM) (Hoagland and Arnon, 1950). Subsequently, the inoculated plants were incubated in a growth chamber with a 12 h dark/ 12 h light photoperiod of 300 µE m^{-2} S^{-1} at 28 ºC and 25 ºC, respectively. The pots were sorted inside the trays containing sterilized riverbed sand on the floor, and irrigation was conducted two times per week by soaking up the sand layer with tap water. The disease severity was visually estimated based on the Percentage of Dried Leaves (PDL= $\frac{Dried\ Leaves}{All\ Leaves} \times 100$) on the stems in the pots on the 30^{th} dpi.

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Table 1 GenBank accession numbers of *Fusarium acuminatum*, other *Fusarium* spp. and *Cylindrocarpon* sp. sequenced by *TEF*1-α used in the phylogenetic analysis of this study.

Species	Isolate	Host/Source	Locality	GenBank Accession No.	Reference
Fusarium acuminatum	PUF035	Unknown	China	HQ165866	Wang *et al*., 2011
F. acuminatum	R-9382	*lavendula* sp.	France	FJ154733	Nalim *et al*., 2009
F. acuminatum	BZ-4	*Lallemantia iberica*	Iran	MZ851155	Current study
F. acuminatum	NRRL 52789	Eggplant soil	Taiwan	JF740857	O'Donnell *et al*., 2012
F. acuminatum	36 HS	*Fagus sylvatica*	Poland	MZ078975	Un published
F. acuminatum	BBA:65106	*Artemisia vulagaris*	Germany	JX397842	Niessen *et al*., 2012
F. acuminatum	WXWH 42	Wheat	USA	MG826892	Funnell-Harris *et al*., 2019
F. acuminatum	WXWH 23	Wheat	USA	MG826875	Funnell-Harris *et al*., 2019
F. tricinctum	XJ-YL-F06-13	Sugar beet	China	KT213355	Un published
F.tricinctum	HL16	Sugar beet	China	KP267446	Un published
F. avenaceum	D32-5	*Hordeum* Sp.	China	KY365602	Un published
F. avenaceum	G17XY4-9	Wheat	China	MG670367	Xu *et al*., 2018
F. arthrosporioides	V I01035	*Hordeum vulgare*	Norway	AJ543510	Kristensen *et al*., 2005
F. arthrosporioides	V I01332	*Linum usitatissimum*	Denmark	AJ543512	Kristensen *et al*., 2005
F. brachygibbosum	ALFBWY137bc21	*Atractylodes lancea*	China	KR108742	Un published
F. brachygibbosum	F 116	Sugar beet	China	KP267360	Un published
F. anguioides	NRRL 31043	Bamboo	China	MH742690	Jacobs-Venter *et al*., 2018
F. anguioides	NRRL 25385	Bamboo	China	MH742689	Jacobs-Venter *et al*., 2018
F. verticilioides	UTFC 2.46	*Pelargonium hortorum*	Iran	MG754391	Un published
F. verticilioides	ITEM 2625	Kernel	Hungary	KF715264	Un published
F. proliferatum	326-EF1	Pineapple	Malaysia	KC584855	Un published
F. proliferatum	327-EF1	Pineapple	Malaysia	KC584857	Un published
F. commune	NT-LH01	*Nicotiana tabacum*	China	MF150040	Un published
F. commune	HMQAU 150044	Kiwifruit	China	KY439903	Yu *et al*., 2017
F. oxysporum	PG 18	Pea	UK	MT630353	Jenkins *et al.*, 2021
F. oxysporum	PG 16	Pea	UK	MT630352	Jenkins *et al*., 2021
Cylindrocarpon sp.	CPC 13545	*Pyrus* sp.	Canada	JF735790	Cabral *et al*., 2012

Results

Morphological identification

The fungus grew relatively slowly (4.2 cm in 7 days) on all plates and produced white-pinkish aerial mycelium with rose to burgundy pigmentation. On the reverse side of the plate, the colony was deep purple. An amber pigmentation was observed in the center of the plate, while at the edge, it was dark tan-coloured (Fig. 2 A). Sporodochia was formed in the centre of the colony in a dark brown mass. On the CLA, macroconidia were formed in the pale orange sporodochia with 3 to 4 × 25 to 35 µm diameter (n = 50). They were falcate, thick-walled, and strongly septate with 3-5 septatae. The apical cell was tapered and relatively elongated, whereas the basal cell was distinctly foot-shaped. Microconidia were rare, 1 or 0-septate, reniform, 2 to 3 × 7 to 15 µm in size (n = 50), and were produced from mono-phialides. Chlamydospores were present (Fig. 2 B-E). According to the morphological studies and classification keys, the fungus was identified as *Fusarium* sp. The established pure culture was deposited in the Culture Collection of DARI

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(CCD-BZ-4) and the Iranian Fungal Culture Collection (IRAN 3997C).

Pathogenicity test

The first symptoms appeared by the seventh dpi in the lower leaves as minor discolouration, and by the 15th dpi, as the symptoms progressed, they became completely yellowed and wilted. By the 30th dpi, the inoculated plants showed a PDL range of 40-100 (Fig. 2 F). Therefore, the same field symptoms appeared in the artificial infection while the negative control remained healthy. Consequently, the same fungus was re-isolated from the inoculated plants, and Koch's postulates were fulfilled.

Molecular identification and phylogenetic analysis

Using Blast search, the sequence of the isolate showed 100% similarity with several ascensions of *Fusarium acuminatum*. The standard 100 top search results aligned from GenBank presented a combination of 83%, 13%, and 4% belonging to *F.acuminatom*, *F.tricinctum*, and *Fusarium* sp., respectively. In the phylogenetic tree, the present fungus was entirely identical for *F.acuminatum* accessions HQ165866, FJ154733, JF740857, MZ078975 and JX397842 with a bootstrap value of 63% (Fig. 3). Accordingly, the morphological characters were confirmed using the molecular and phylogenetic approaches.

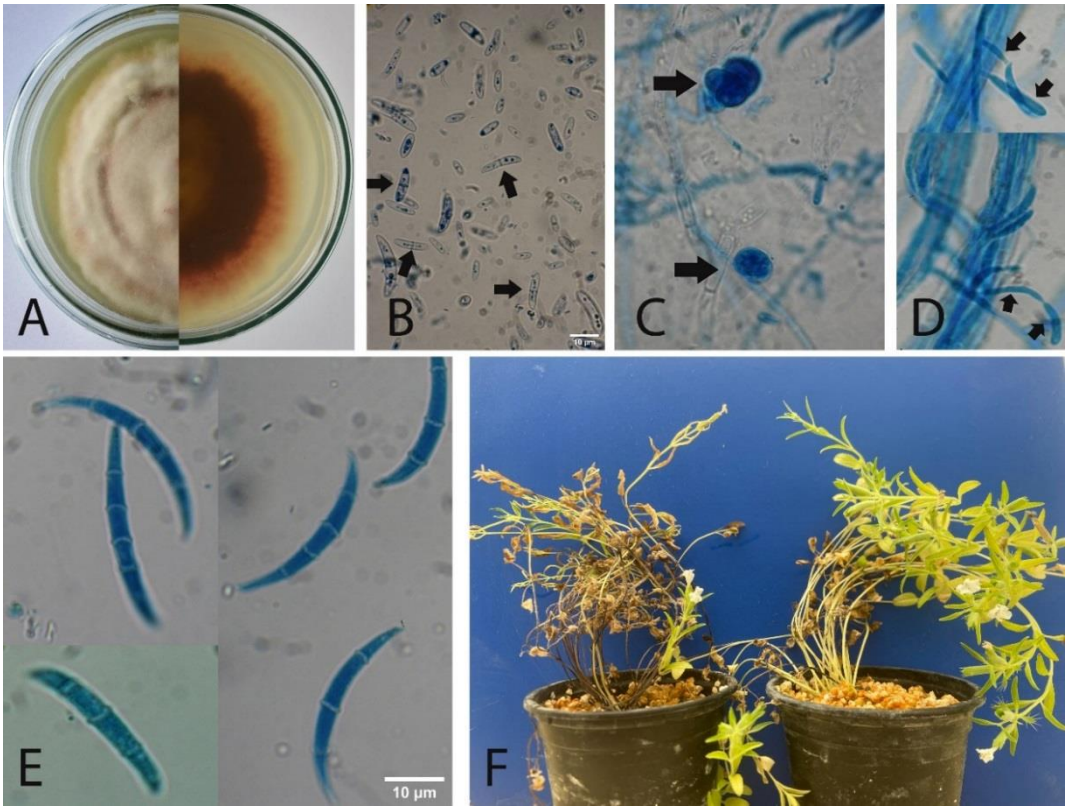

Figure 2 Colony shape, microscopic characters and pathogenicity test of *F. acuminatum* derived from Dragon's head *Lallemantia iberica*. (A) Colony on PDA at 15 dpi in 25 ºC, left and right hand semi plates show habit of fungus growth from the top and bottom sides, respectively, (B) 1-septate, reni-form microconidia indicated with arrows, are distinguished from 0-septate micro-conidia, (C) Arrows indicate cluster and singular chlamydospores, (D) Macroconidia prodced on mono-phialides growing from the hyphae, (E) 3-5 septate macro-conidia, (F) Disease severity measured by PDL (left and right pots with 100 and 40 PDL, respectively) in the glasshouse at 30 dpi. Scale bar: 10µm and all on Carnation Leaf Agar (CLA) (B-E).

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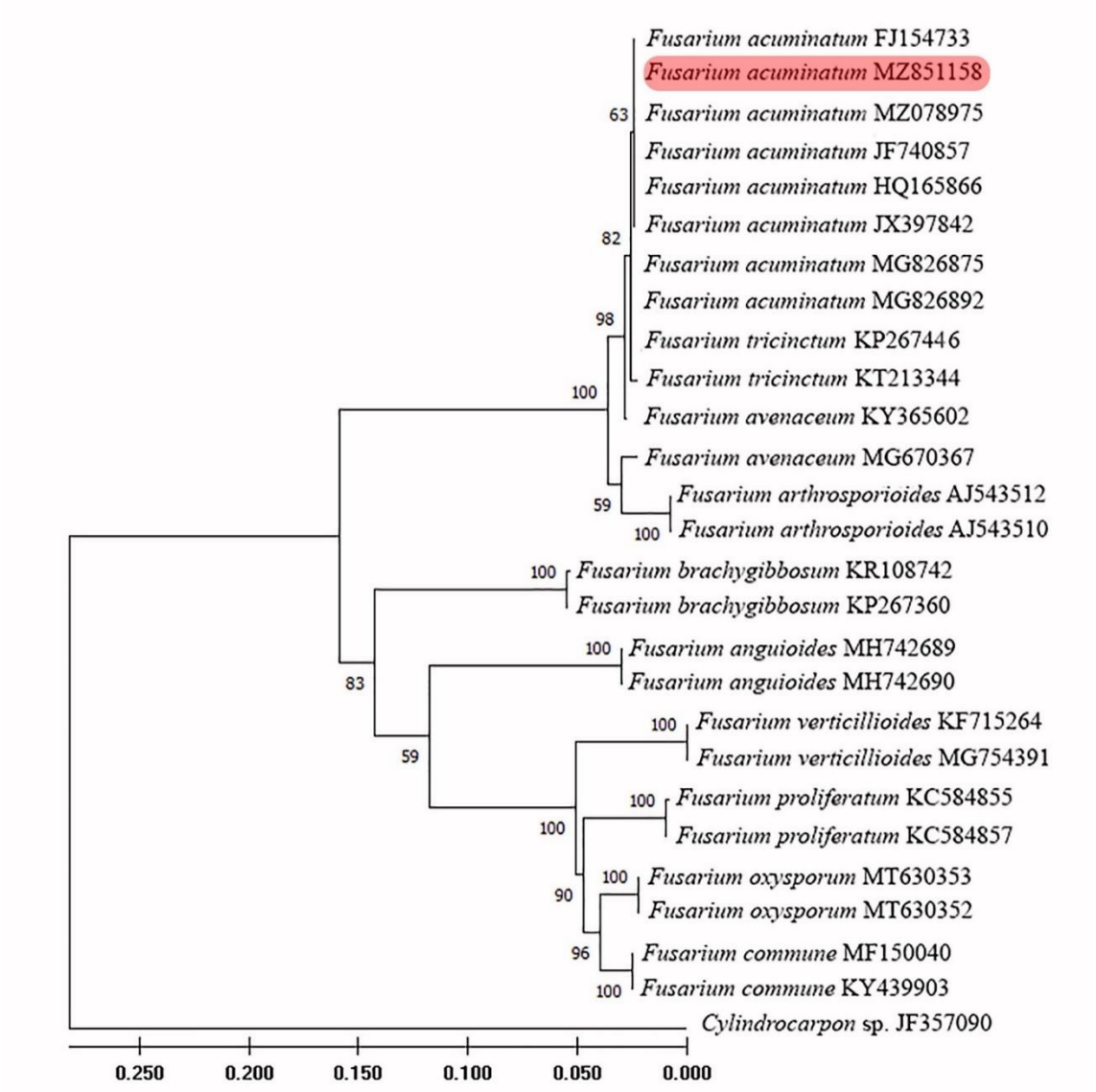

Figure 3 Maximum likelihood tree of *Fusarium acuminatum* MZ851158 (in the red box) isolated from Dragon's head (*Lallemantia iberica*) in Iran and other *Fusarium* spp. generated based on *TEF*1-α sequences. *Cylindrocarpon* sp. is used as an outgroup. The bootstrap values higher than 50% are displayed, and the number of substitutions is shown in the scale bar.

Discussion

The filamentous fungus *Fusarium* Lonk (Nectriaceae, Hypocreales) is a soil-borne cosmopolitan genus that can survive on a wide range of substrates and can live as a saprophyte on organic debris or in association with plants as a pathogen or endophyte (Leslie and Summerell, 2006; Zakaria and Ning, 2013). The results obtained in this research revealed that the fungal agent causing yellows and wilting on the crop is *Fusarium acuminatum* Ellis & Everh. sensu Gordon. This species has a cosmopolite distribution able to attack leaves and roots, causing different diseases, including leaf dieback, light brown lesions, and root rot on

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more than 700 different host plant species (Farr and Rossman, 2020). Some of the recent economically important reports of *F. acuminatum* are pea root rot from Canada (Safarieskandari *et al.*, 2020), garlic bulb rots in Serbia (Ignjatov *et al.*, 2017), postharvest rot on stored kiwifruit from China (Wang *et al*., 2015), damping-off on Aleppo pine in Algeria (Lazreg *et al*., 2014), leaf spot on onion from Georgia (Parkunan and Ji, 2013); chlorosis, dropping and rolling of the leaves of pigeon pea from India (Sharma *et al*., 2014), and wilt on sunflower from USA (Mathew *et al.*, 2010). Of these reports, only sunflowers wilt (Mathew *et al*., 2010) and pigeon pea leaf rolling (Sharma *et al*., 2014) resemble the current study where the fungus was isolated from stems of Dragon's head and the disease dominantly appeared as chlorotic, rolled, yellowed, and wilted leaves. In Iran, the fungus has been isolated from several crops such as lentils (Torbati *et al*., 2019), sesame, sunflower, watermelon, citrus, cucumber, alfalfa (Gerlach and Ershad, 1970), onion (Sobhani *et al*., 2019), peanut (Pourabdollah and Ershad, 1997), sugar beet (Dastjerdi *et al.*, 2003), barley and wheat (Darvishnia *et al.*, 2006). According to literature (Ershad 2009; Farr and Rossman, 2020), this is the first report of *F.acuminatum* on Dragon's head in Iran and the world. This study characterised a very destructive disease that could cause significant yield loss of the crop. In the years 2020 and 2021, the disease was observed in most fields in the North West of Iran, which shows its wide distribution and great importance in the country. This finding could impose new regulations on the disease management protocols of the crop in Iran. Moreover, the breeding programs of Dragon's head must be adopted to evaluate its germplasm against the pathogen.

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Statement of Conflicting Interests

The authors state that there is no conflict of interest.

Authors' Contributions

The authors' contribution is 100%.

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قارچ *Fusarium acuminatum*: یک بیمارگر جدید و عامل زردی و پژمردگی در بالنگوی شهری (*Lallemantia iberica*) در ایران

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چکیده: بالنگوی شهری *Lallemantia iberica* در حال حاضر به‌عنوان یک دانه روغنی در مناطق دیم ایران کشت می‌شود. در سال ۱۳۹۸ علائمی ازجمله گیاهچه‌میری، زردی و پژمردگی روی گیاهان در یک مزرعه تجاری در شهرستان مراغه مشاهده شد. براساس خصوصیات مورفولوژیکی و مولکولی، قارچ جدا شده از گیاهانِ دارای علایم بیماری، به‌عنوان *Fusarium acuminatum* شناسایی شد. بیماری‌زایی قارچ از طریق مایه‌زنی گیاه میزبان تأیید شد. متعاقباً، مفروضات کخ با جداسازی مجدد همان قارچ از گیاه آلوده مایه‌زنی شده محقق شد. این اولین گزارش از بیماری پژمردگی فوزاریومی در بالنگوی شهری در ایران و جهان است.

واژگان کلیدی: Translation elongation factor 1-a، پژمردگی و زردی، از پاافتادگی، دانه روغنی، سبب‌شناسی قارچ، مراغه

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