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Morphological and molecular characterization of a novel *Pestalotiopsis trachycarpicola*, causing Garden Croton leaf spot in Iran

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Abstract: In the spring of 2021, leaf spot disease symptoms were observed in the Garden Croton (*Codiaeum variegatum*) plants in one of the ornamental plant greenhouses of Mahallat County, Markazi Province, Iran. To identify the causal agent of the disease, infected plant leaves sampled and then were transferred to the mycology laboratory and nine fungal isolates were recovered. The fungal isolates were characterized based on the morphological features and molecular data using the combined sequences of the internal transcribed spacer (ITS) regions and the translation elongation factor 1-alpha (*tef*) gene for a representative isolate UT2022. According to the morphology and phylogenetic analysis, the isolate UT2022 was identified as *Pestalotiopsis trachycarpicola*. The pathogenicity of the isolate UT2022 was performed on healthy and growing leaves of the *C. variegatum* plants. Inoculated leaves showed leaf spot symptoms 14 days after the inoculation, while the leaves of the control plants were symptomless. To complete Koch's postulate, *P. trachycarpicola* was re-isolated from the newly produced leaf spots. Based on the bibliography, *P. trachycarpicola* is reported as a new taxon for fungi of Iran, as well as *P. trachycarpicola* is reported as the causal agent of the leaf spot symptom of the *C. variegatum* in the world.

Keywords: *Codiaeum variegatum*, fungal disease, ITS, *tef*, pathogenicity

INTRODUCTION

The Garden Croton is a beautifully variegated leafy tropical perennial plant with glabrous branches and ovate to linear leaves (Dutta, 2004). Garden Croton has been widely cultivated in the ornamental greenhouses of Iran. Indoor plants, like other plant taxa, are attacked by various plant pathogens. Fungi are the most common microorganisms attacking plants and cause severe diseases at different developmental stages. Several fungal species have been reported to occur on this host namely *Nectriella pironii* Alfieri & Samuels (causal agent of stem gall disease), *Dietelia codiaei* (Syd.) Boerema (causal agent of rust disease), *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc., *Corynespora cassiicola* (Berk. & M.A. Curtis) C.T. Wei (causal agents of leaf spot disease) and *Pseudoidium neolycopersici* (L. Kiss) L. Kiss (causal agents of powdery mildew disease). According to the studies done throughout the world, different species of *Alternaria* Nees, *Ascochyta* Lib., *Botryodiplodia* Sacc., *Botrytis* P. Micheli, *Cercospora* Fresen. ex Fuckel, *Cladosporium* Link, *Coniothyrium* Corda, *Corticium* Pers., *Diaporthe* Fuckel, *Dietelia* Henn., *Diplodia* Fr., *Dothiorella* Sacc., *Fusarium* Link, *Gloeosporium* Desm. & Mont., *Glomerella* Spauld. & H. Schrenk, *Guignardia* Viala & Ravaz, *Helminthosporium* Link, *Leptosphaeria* Ces. & De Not., *Macrophomina* Petr., *Nectria* (Fr.) Fr., *Phyllosticta* Pers., *Pythium* Nees, *Phytophthora* de Bary, *Rhizoctonia* DC., *Verticillium* Nees, *Sclerotium* Tode have been reported as the causal agents associated with *C. variegatum* plant (Farr and Rossman, 2022). Based on the importance of the Garden Croton as an ornamental plant in Iran, the main aim of the present research was the characterization of the causal agent of the leaf spot disease in *C. variegatum* based on morphology and phylogenetic criteria.

MATERIALS AND METHODS

Isolation and morphology

In the spring of 2021, leaf spot disease symptoms were observed in growing leaves of Garden Croton (*Codiaeum variegatum* L.) in one of the ornamental plant propagation greenhouses (33°52'49.0"N

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50°28'48.2"E) of Mahallat City, Markazi Province, Iran. Plants showing typical leaf spot symptoms were collected and transferred to the mycological laboratory of the University of Tehran. Fungal isolates were recovered using the procedure provided by Refaei et al. (2011) with some minor modifications. Infected plant tissues were cut into small pieces, and portions of leaves with characteristic spots were surface sterilized in 2% sodium hypochlorite (NaOCl) solution for 1 min, and then with 70% ethanol for 1 min and at the end rinsed twice with sterile distilled water. Then, disinfested plant pieces were dried with a sterile paper towel and placed on a 2% water agar (WA), and inoculated eight centimeter in diameter Petri dishes were kept at 25ºC in the continuous dark condition for five days. Nine morphologically similar isolates were recovered from the ten infected leaves and the fungal colonies were purified by the hyphal tip method and the resulting hyphal tips were placed on the potato dextrose agar (PDA). The Petri dishes were kept for seven and fourteen days at 25°C under alternating near-UV light (12 h light/12 h dark) for further study. Morphological features of the fungal isolates were investigated based on available references such as Zhang et al. (2021). The recovered isolate (UT2022) was deposited in the Agricultural Microbial Collection of the Agricultural Biotechnology Research Institute of Iran, Karaj, Iran (ABRII), with accession no. of ABRIICC 10359.

PCR amplification and phylogenetic analysis

DNA was extracted from the growing seven days old fungal mycelium (isolate UT2022) using the protocol of Zhong and Steffenson (2001). The whole internal transcribed spacer (ITS1-5.8S-ITS2) regions of the rDNA and the partial translation elongation factor 1-alpha (*tef*) genes were amplified by use of ITS1 (TCCGTAGGTGAACCTGCGG) and ITS4 (TCCTCCGCTTATTGATATGC) (White et al. 1990), and EF1-728F (CATCGAGAAGTTCGAGAAGG) and EF1-986R (TACTTGAAGGAACCCTTACC) (Carbone and Kohn, 1999) primers. PCR amplification was carried out with the final volume of 25 µl having 10 µL of *Taq* DNA polymerase Mix Red–Mgcl2, 11 µL deionized water, 1 µL of each primer (10 pmol), and 2 µL of template DNA. The PCR amplifications were done using a thermocycler with the following thermal conditions, for ITS: initial denaturation at 94ºC for 3 min, followed by 35 cycles of denaturation step at 94ºC for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s, and terminated with a final extension step at 72°C for 10 min, and for *tef*: initial denaturation at 94 °C for 8 mins, and then followed by 35 cycles each with denaturation at 94 °C for 15 s, annealing at 55 °C for 20 s and the extension at 72 °C for 1 min, and a final extension at 72 °C for 5 min .The PCR end products were resolved in 1.5% agarose gel by electrophoresis using 1X Tris-Boric acid-EDTA buffer (TBE) and the PCR products were sent to Cardiogenetic Research Center (Rajae hospital, IRAN) for sequencing. The newly generated sequences were manually edited with Chromas 2.6.6 software (Technelysium, Australia) and the edited sequences were used in the phylogenetic analysis. The resulting sequences of ITS and *tef* were all subjected to a BLAST search (Altschul et al. 1990) to compare the obtained sequences with the most similar sequences in the National Centre of Biological Information (NCBI). The edited sequences of our isolate were aligned with those sequences mostly obtained from the Silva et al. (2020) research in Clustal W (Thompson et al. 1994) software. Thirty-three ITS and *tef* reference sequences of *Pestalotiopsis*, *Pseudopestalotiopsis* Maharachch., K.D. Hyde & Crous, *Neopestalotiopsis* Maharachch., K.D. Hyde & Crous, *Seiridium* Nees, and *Seimatosporium* Corda species and also sequences of *Broomella vitalbae* as out-group taxon were selected for phylogenetic analyses (Table 1.). The maximum likelihood (ML) analysis (Felsenstein 1973) was performed with heuristic search using MEGA X (Kumar et al. 2018). Bootstrap analysis (Felsenstein 1985) of the obtained ML tree was done with 1000 replicates.

Pathogenicity

Pathogenicity tests were conducted in the spring of 2021 in the greenhouse of the Department of Plant Protection, University of Tehran, Iran. Wound inoculation of the plant leaves was done by the protocol of Wikee et al. (2013) with some modifications. Pathogenicity tests were done in four replicates and one control. For inoculation, healthy and growing leaves of the plant (*C. variegatum*) were surface sterilized using the 70 % ethanol and then washed three times with sterile distilled water. The leaves were dried with sterile tissue paper. Mycelial plugs (5 mm) were taken from the leading margins of the seven days old fungal colonies and placed on the wounds upside down to facilitate the contact of fungal mycelium with the wounds. In the control, wounded leaves were inoculated with mycelium-free PDA plugs using the same procedure. All inoculated plants were placed on a greenhouse bench and were kept at 20 °C until the development of potential disease symptoms. Two weeks after inoculation, the fungal isolate was re-isolated from the inoculated leaves having leaf spot symptoms and then was cultured on PDA. The morphological features of the re-isolated strain were compared to those of the original recovered isolate, fulfilling Koch's postulates.

RESULTS

Symptoms on the plant in nature

Small necrotic reddish-brown spots have initially appeared on the older leaves. Then, the spots were enlarged and became elliptic to irregular in shape and pale brown in the center. At the center of the spots, the pycnidia are produced. Over the extended periods of disease development, in small leaves, spots may coalesce to form large and necrotic areas and may cause the death of the infected leaves.

Table 1. Used sequences of *Pestalotiopsis* and related genera in the phylogenetic analysis. Newly generated sequence is in boldface.

Species	Strain	Host/Source	Country	GenBank Accession no.	
				ITS	*tef*
Pestalotiopsis trachycarpicola	**UT2022**	***Codiaeum variegatum***	**Iran**	**ON325801**	**ON411799**
	IFRDCC 2440	*Trachycarpus fortunei*	China	JQ845947	JQ845946
Pestalotiopsis australasiae	CBS 114141	*Protea* sp.	Australia	KM199298	KM199501
	CBS 114126	*Knightia* sp.	New Zealand	KM199297	KM199499
Pestalotiopsis biciliata	CBS 236.38	*Paeonia* sp.	Italy	KM199309	KM199506
	CBS 124463	*Platanus* × *hispanica*	Slovakia	KM199308	KM199505
Pestalotiopsis rhizophorae	MFLUCC 17-0416	*Rhizophora apiculata*	Thailand	MK764283	MK764327
Pestalotiopsis parva	CBS 114972	Leaf	Hong Kong	MH553980	MH554397
	CBS 278.35	*Leucothoe fontanesiana*	Thailand	KM199313	KM199509
Pestalotiopsis humicola	CBS 115450	*Ilex cinerea*	China	KM199319	KM199487
	CBS 336.97	soil in tropical forest	New Guinea	KM199317	KM199484
Pestalotiopsis adusta	CBS 263.33	*Rhododendron ponticum*	Netherlands	KM199316	KM199489
	ICMP 6088	Refrigerator door PVC gasket	Fiji	JX399006	JX399070
Pestalotiopsis portugallica	CBS 684.85	*Camellia japonica*	New Zealand	MH554065	MH554501
	CBS 393.48	Unknown	Portugal	KM199335	KM199510
Pestalotiopsis camelliae	CBS 443.62	*Camellia sinensis*	Turkey	KM199336	KM199512
	MFLUCC 12-0277	*Camellia japonica*	China	JX399010	JX399074
Pestalotiopsis pini	MEAN 1095	*Pinus pinea*	Portugal	MT374682	MT374695
	MEAN 1094	*Pinus pinea*	Portugal	MT374681	MT374694
Pestalotiopsis rhododendri	IFRDCC 2399	*Rhododendron sinogrande*	China	KC537804	KC537811
Pestalotiopsis chamaeropis	CBS 186.71	*Chamaerops humilis*	Italy	KM199326	KM199473
	CBS 113607	Unknown	Thailand	KM199325	KM199472
Pestalotiopsis australis	MEAN 1096	*Pinus pinea*	Portugal	MT374684	MT374696
	MEAN 1109	*Pinus pinea*	Portugal	MT374679	MT374692
Pseudopestalotiopsis theae	MFLUCC12-0055	*Camellia sinensis*	Thailand	JQ683727	JQ683743
Neopestalotiopsis aotearoa	HNPeHNLD2002	*Hevea brasiliensis*	China	MT764948	MT800517
	HNPeHNLD2001	*Hevea brasiliensis*	China	MT764947	MT800516
Seiridium camelliae	CBS 910.85	*Cupressus sempervirens*	South Africa	LT853065	LT853162
	CBS 909.85	*Cupressus lusitanica*	South Africa	LT853064	LT853161
Seimatosporium vitis-viniferae	CRCC 214	*Vitis vinifera*	Italy	MN862461	MN862447
	CRCC 212	*Vitis vinifera*	Italy	MN862459	MN862445
Seimatosporium pistaciae	CPC 24457	*Pistacia vera*	Iran	MH554126	MH554561
	CBS 138865 HT	*Pistacia vera*	Iran	KP004463	MH554432
Broomella vitalbae	HPC 1154	Unknown	China	MH554173	MH554608

Taxonomy and phylogenetic analysis

After fourteen days of incubation, a fungal isolate with exact morphological characteristics of *Pestalotiopsis* Steyaert was identified based on the conidia and pycnidia features in the culture medium. Based on the results of the morphological investigation, nine identical fungal isolates recovered from the disease symptoms. The isolate UT2022 was used as a representative isolate. Under the *in vitro* condition, the colony of the isolate on PDA was white, and fluffy with an undulated edge, and fast-growing, and reaching 70 mm in diameter after seven days incubation at 25°C under the alternating near-UV light (12 h light/12 h darkness). Mycelium was hyaline, smooth, and septate, 2.5–5 µm in diameter. Conidiomata were pycnidial, globose, scattered, semi–immersed, and black, with globose and black conidial masses, 100 – 550 µm diameter. Conidiophores were reduced to conidiogenous cells, lageniform to subcylindrical, discrete, smooth, thin-walled, hyaline, with 2-4 proliferations, 8–15.5×2.5–3 µm ($\bar{x}$= 12 × 3 µm, n = 20). Conidia fusoid, straight to slightly curved, olivaceous to yellow, 4-septate, 20-25.5 × 5.5-7 µm ($\bar{x}$ =23 × 6.0 µm, n = 50), thin-walled, and verruculose wall, with the basal cell obconic with truncate base, thin-walled, hyaline, 3–5 µm long ($\bar{x}$= 4 µm). Three median cells were doliiform, with thick granular walls, 12–16 µm long, constricted at the septa, concolorous, pale-yellow to brown, with darker septa. Apical cell conical, hyaline, with smooth wall, 2.8–5.5 µm long ($\bar{x}$= 4 µm), having 3–4 concurrent tubular apical appendages (mostly 3), filiform, unbranched, and arising from the apical crest, 9–19 µm long ($\bar{x}$= 14 µm). Single basal appendage was straight, 3–7 µm long ($\bar{x}$= 4 µm). Sexual morph was not observed (Fig. 1 A-D). These morphological characteristics of the obtained isolate matched well with the description provided by *P. trachycarpicola* Yan M. Zhang & K.D. Hyde (Zhang et al. 2012). Since the ITS sequence data alone is not sufficient to resolve the species in the genus *Pestalotiopsis* (Tempesta et al. 2011; Maharachchikumbura et al. 2014).

We used two different sequence data for species delineation. The results of the phylogenetic evaluation based on the combined sequences of the ITS regions and the *tef* gene revealed three main clades in the phylogenetic tree. The species of *Pestalotiopsis* were placed in the topmost clade with a 95% bootstrap value. The newly obtained isolate (UT2022) was clustered with another isolate of *P. trachycarpicola* (IFRDCC 2440) from GenBank with the maximum bootstrap support of 100% (Fig. 2). The new sequences were deposited in the GenBank (NCBI) with accession numbers of ON325801 and ON411799 for ITS *tef*, respectively.

Pathogenicity test in the greenhouse

Pathogenicity of the recovered isolate UT2022 was performed on leaves of *C. variegatum.* Two weeks after the artificial inoculation in the greenhouse, leaf spot symptoms approximately 2–3 cm in diameter on inoculated leaves were observed. The spots were nearly circular. First, they appeared as small red spots with white to gray at the center, which developed and became purple to dark reddish-brown and symptoms were similar to the leaf spots observed in naturally-infected plants. The causal fungus was re-isolated with frequency of about 90% from the artificially inoculated leaves with leaf spot symptoms on PDA and was morphologically compared with the original fungal isolate that was obtained from field leaf spot symptoms (Fig 1. E-F). Two weeks after inoculation, no symptoms appeared on control plants.

DISCUSSION

Plant growth and yield are negatively affected by different biotic and abiotic stresses. Ornamental and wild plants may have an important effect in the spread of diseases and maintaining pathogens. In this study, a fungal isolate with similar characteristics to *Pestalotiopsis* was recovered from Garden Croton with leaf spot symptoms.

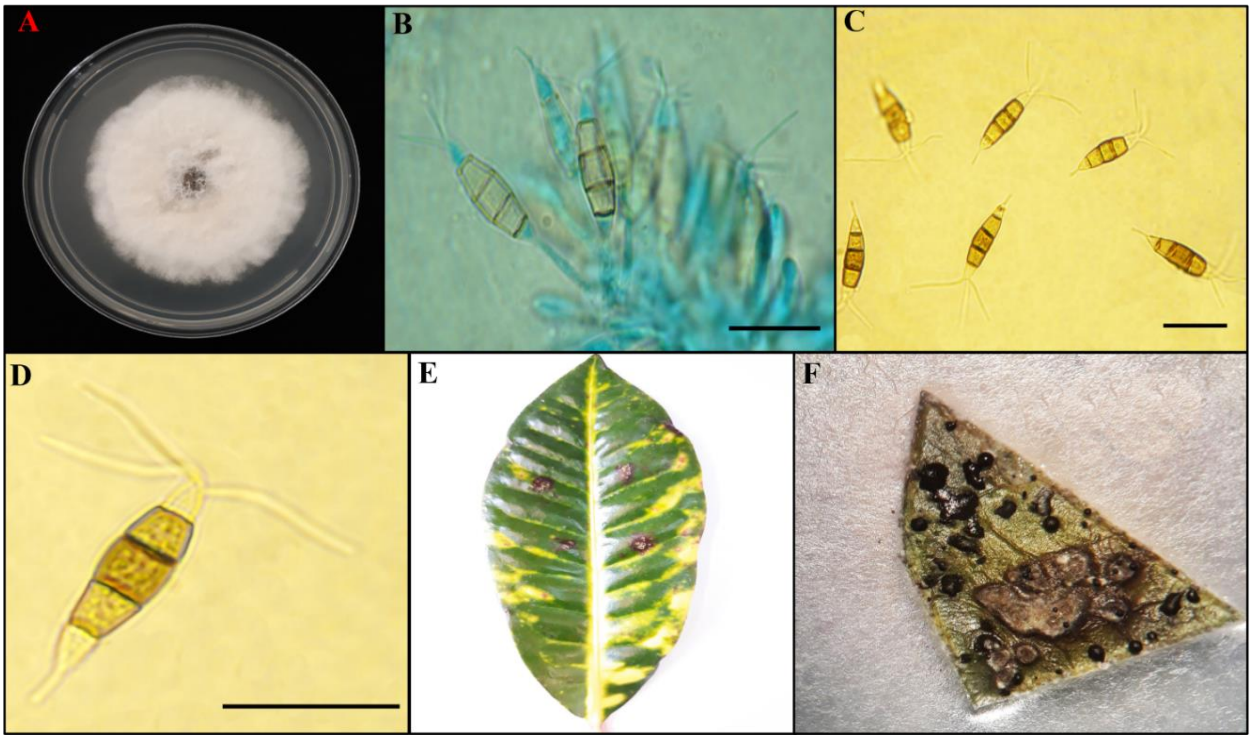

Figure 1. Culture characteristics, morphology and pathogenicity test of *Pestalotiopsis trachycarpicola* isolate UT2022: (A) Colony on potato dextrose agar after incubation for seven days at 25 °C, (B) Conidiophores and conidia; (C-D) Conidia, (E) Leaves of *Codiaeum variegatum*, showing leaf spot symptom caused by *P. trachycarpicola* at 14th day after artificial inoculation and (F) Pycnidia formed on the surface of inoculated leaves in the greenhouse. Scale bars = 20 µm.

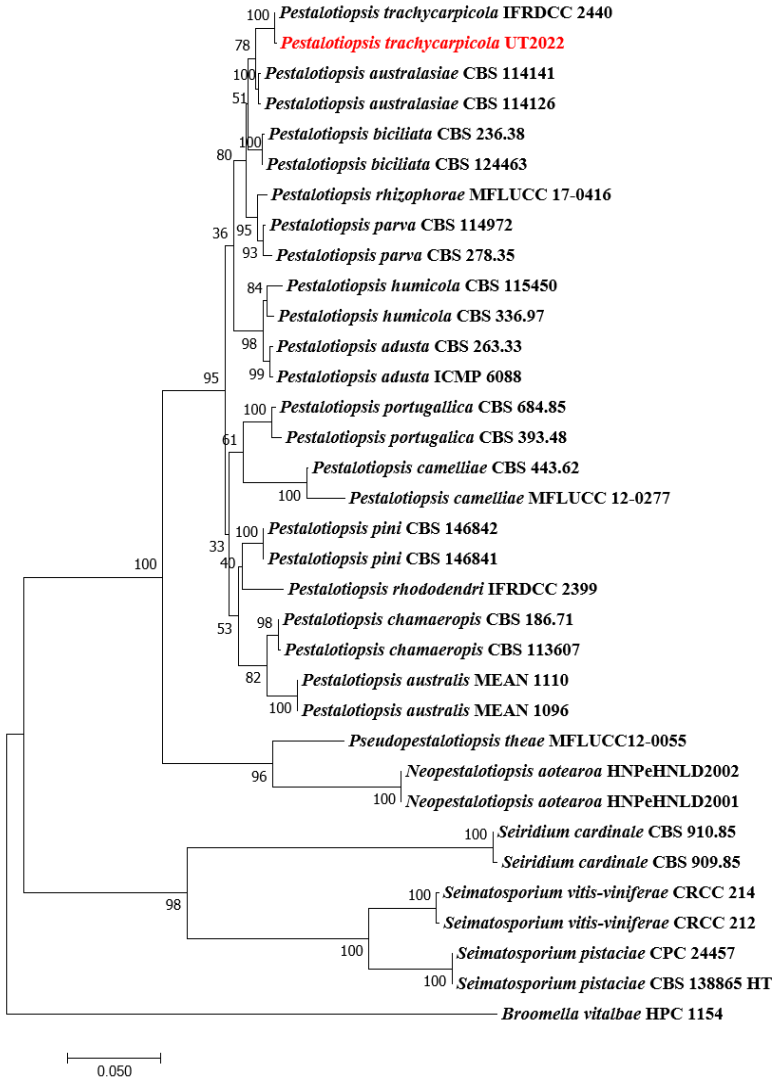

Fig. 2. Maximum likelihood (ML) tree generated in MEGA X, based on the aligned sequence data of the ITS regions and *tef* gene of the 33 isolates of *Pestalotiopsis*, *Pseudopestalotiopsis*, *Neopestalotiopsis*, *Seiridium*, *Seimatosporium* and *Broomella vitalbae* HPC 1154 was chosen as the outgroup taxon. Bootstrap values (1000 replicates) indicated at the nodes. Strain in red was isolated in this study. The scale bar indicates expected nucleotide changes per site.

The genus *Pestalotiopsis* (Pestalotiopsidaceae, Amphisphaeriales, Sordariomycetes), was first introduced by Steyaert in 1949, is a species-rich asexual genus identified by moderately fusiform conidia, each with a basal hyaline cell, and three pigmented median cells, and also an apical hyaline cell with two or even more apical appendages that are widely distributed in the tropical and temperate regions (Das et al. 2021). Species of *Pestalotiopsis* are important and common pathogens of plants that cause various diseases, such as leaf spot, shoot dieback, fruit rot, and various post-harvest diseases (Li et al. 2021). Recent studies have shown that most of the introduced phylogenetic species in the genus *Pestalotiopsis* can be identified based on the combined sequence data of the ITS-rDNA, b-tubulin (*tub2*), and *tef*. In the present study, we used the ITS-rDNA and the *tef* gene sequences in the phylogenetic analysis for species identification, in addition to the morphological features. The isolate UT2022 was identified as *P. trachycarpicola*.

Pestalotiopsis trachycarpicola was first identified and described as a causal agent of leaf spot disease on *Trachycarpus fortunei* (Hook.) H.Wendl. in China (Zhang et al. 2012). Later, it was reported as a causal agent of the leaf spot on *Sorghum bicolor* (L.) Moench (Fan et al. 2021) and leaf spots on *Gentiana rhodantha* Franch. in China (Zhang et al. 2021). Currently, more than 10 species of *Pestalotiopsis*, including *Pestalotiopsis acaciae* (Thüm.) K. Yokoy. & S. Kaneko from Diospyros lotus L., *Pestalotiopsis brassicae* (Guba) Maharachch., K.D. Hyde & Crous

from *Ulmus carpinifolia* var. *umbraculifera* (Trautv.) Rehder, *Pestalotiopsis citri* (Mundk. & Khesw.) Y.X. Chen from *Citrus aurantinum* L., *Citrus limettioides* Tanaka and *Citrus unshiu* Marcow., *Pestalotiopsis disseminata* (Thüm.) Steyaert from *Feijoa sellowiana* Berg, *P. longiseta* (Speg.) K. Dai & Tak. Kobay. from *Actinidia chinensis* Planch., and *Camellia sinensis* (L.) Kuntze, *P. funerea* (Desm.) Steyaert from *Cedrus deodara* (Rob. ex Lambert) G. Don, *Camellia sinensis*, *Cupressus arizonica* Greene, *C. sempervirens* L., *Picea abies* Degen, *Prunus* sp., *Sequoia sempervirens* Endl., *P. macrospora* (Ces.) Stey. from *Corylus avellana* L., *P. guepinii* (Desm.) Stey. from *Cyperus rotundus* L., *P. neglecta* (Thuem.) Stey. from *Euonymus japonicus* L., *P. longisetula* (Guba) X.A. Sun & Q.X. Ge from *Fragaria ananassa* Duchesne, *P. smilacis* (Schw.) Sutton, *Smilax* sp., *P. uvicola* (Speg.) Bissett from *Vitis vinifera* L., *P. theae* (Sawada) Stey. *Camellia sinensis*, and *Musa* × *paradisiaca* L., *P. nattrassii* Stey. from *Camellia sinensis* have been reported from Iran (Ershad 2022). Based on the literature review, this is the first report of *P. trachycarpicola* for the fungi of Iran. Furthermore, *P. trachycarpicola* previously had not been reported from the *Codiaeum variegatum*. After the pathogenicity test and observed newly produced symptoms on the inoculated leaves, we report for the first time that *P. trachycarpicola* is the causal agent of the leaf spot disease on *C. variegatum* and it should be considered as a novel disease of the Garden Croton in the world. Furthermore, since Garden Croton and other Euphorbiaceae plants are grown annually in ornamental plant greenhouses in different regions of Iran, more relevant studies should be done in the different area to reveal the species diversity and richness for precise planning the efficient plant disease management programs.

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خصوصیات ریخت شناختی و مولکولی گونه *Pestalotiopsis trachycarpicola* عامل لکه برگی کروتون باغی در ایران

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چکیده : در بهار سال ۱۴۰۰ در یکی از گلخانه‌های پرورش گل زینتی در شهرستان محلات از استان مرکزی علایم بیماری لکه برگی روی گیاه کروتون باغی (*Codiaeum variegatum*) مشاهده شد. به منظور شناسایی عامل ایجاد کننده بیماری، از برگ های آلوده نمونه برداری انجام شد و نمونه ها به آزمایشگاه منتقل گردیدند. در نهایت نُه جدایه قارچی با جدایه UT2022 به عنوان جدایه نماینده به دست آمدند. براساس ویژگی‌های ریخت شناختی و داده‌های مولکولی حاصل از ترکیب توالی نوکلئوتیدی نواحی ژنومی ITS و *tef* جدایه نماینده به عنوان *Pestalotiopsis trachycarpicola* شناسایی گردید. آزمون بیماری‌زایی روی برگ‌های سالم و در حال رشد گیاه میزبان انجام شد. علایم لکه برگی پس از ۱۴ روز روی گیاهان مایه زنی شده ظاهر گردید. در حالی که در گیاهان شاهد علایم بیماری ظاهر نشد. به منظور تکمیل اصول کخ، قارچ عامل بیماری دوباره از علایم لکه برگی جدید جداسازی گردید. طبق بررسی منابع، گونه *P. trachycarpicola* به عنوان آرایه جدید برای قارچ‌های ایران معرفی می‌شود و همچنین گونه *P. trachycarpicola* به عنوان عامل بیماری لکه برگی گیاه *C. variegatum* در دنیا گزارش می‌شود.

کلمات کلیدی: *Codiaeum variegatum*، بیماری قارچی، ITS، *tef*، بیماری زایی

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