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Research Article

Polyphasic characterization and pathogenicity of *Colletotrichum theobromicola* on pomegranate (*Punica granatum*) in Iran

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Keyword

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Abstract

Pomegranate (*Punica granatum*) is nourishing fruit with several useful medicinal and health properties. Fruit rot diseases are among the main constrains for pomegranate production worldwide, which may occur in orchards or as post-harvest diseases during storage. Anthracnose disease caused by *Colletotrichum* species is one of the important diseases of pomegranates in many pomegranate producing countries; however, there is a paucity of knowledge on the occurrence and the identity of *Colletotrichum* spp. causing anthracnose disease on pomegranate in Iran. In recent years, there has been a significant prevalence of pomegranate fruit rot symptoms, resembling anthracnose disease in the pomegranate orchards of Golestan province. Therefore, the current research was aimed to characterize the causal agent of the disease and also evaluate their pathogenicity on pomegranate fruit. For this purpose, sampling was done from the pomegranate orchards of Golestan province during 2021 growing season. Isolation and purification of fungal isolates was done using common methods in plant pathology on acidified potato dextrose agar (PDA) culture medium. The isolates were identified by combining morphological traits with sequence data of the ITS-rDNA genomic region and *beta-tubulin* (*BUT*) gene. By combining morphological data with molecular data, the isolates were identified as *Colletotrichum theobromicola* (*C. gloeosporioides* species complex). The results of pathogenicity tests on pomegranate fruit in laboratory conditions showed that the mentioned isolates caused symptoms five days after inoculation. Even though several *Colletotrichum* species in *C. gloeosporioides* and *C. acutatum* species complexes are known to cause fruit anthracnose and leaf blight symptoms on pomegranate worldwide, in the present study *C. theobromicola* was isolated as the sole causal agent of the disease for the first time in Iran. Geographical distribution, economic impact and ecology of *Colletotrichum* spp. in pomegranate producing areas in Iran remain to be studied.

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Introduction

Pomegranate is an economically important perennial fruit crop, with ancient domestication and cultivation history, backs to over 5 000 years (Chandra *et al.* 2010). The center of origin and domestication of pomegranate is the mainland of Iran and neighboring countries from where it speared to India, China and the Mediterranean region and subsequently in the 1700's it was introduced to North America (Chandra *et al.* 2010). Currently pomegranate is cultivated in many countries worldwide (Derakhshan *et al.* 2018). Iran ranks among the major pomegranate producing countries in the world with an area of 60,000 ha under pomegranates cultivation and annual production of 650,000 tons, accounts for 75% of world pomegranate production (Mirabolfathy *et al.* 2012, Golmohammadi *et al.* 2020). Pomegranate orchards are distributed across the country, encompassing diverse commercial cultivars (Derakhshan *et al.* 2018). Considering the undemanding nature of pomegranate and its adoptability for unfavorable climatic conditions (including drought tolerance, thriving well in dry conditions with marginal soils of low fertility and low water), pomegranate is a suitable candidate for replacing demanding fruit tree orchards in many regions of Iran. In this regard the policy of government is to encourage pomegranate cultivation by establishing new orchards and also increase production yield.

Several biotic and abiotic factors adversely affect pomegranate longevity and production yield worldwide (Phillips *et al.* 2013; Úrbez-Torres *et al.* 2017; Golmohammadi *et al.* 2020). Foliar diseases caused by bacteria and fungi such as bacterial blight (*Xanthomonas axonopodis* pv. *punicae*), leaf and fruit spot (*Pseudocercospora punicae*) and anthracnose disease (*Colletotrichum spp*.) are among the economically important diseases of pomegranate in many countries (Munhuweyi *et al.* 2016; Xavier *et al.* 2019). Fruit rot and spot diseases are responsible for significant crop losses in garden or after harvest during storage. *Elsinoe punicae* (Bitanc. & Jenkins, 1940) the causal agent of pomegranate scab disease mainly affects fruits from the early stages of flowering until fruit maturing in orchards, which results in significant yield loss and also reduces commercial value of the fruit (Carstens *et al.* 2018). Other fungal species including *Alternaria alternata* (Fr.) Keissl., *Aspergillus niger* Tiegh., *Aureobasidium pullulans* (de Bary & Löwenthal) G. Arnaud, *Botrytis cinerea* Pers., *Cladosporium cladosporioides* (Fresen.) G.A. de Vries, *Penicillium* spp., *Pilidiella granati* (Sacc.) Aa mainly induce internal rot on fruits in garden or as post-harvest during storage (Mincuzzi *et al.* 2022). Fruit rot agents penetrate the fruit via natural openings or physical injuries made by biotic or abiotic factors. Fruit rot symptoms are widespread in orchards where poor pest management strategies and horticultural principles are practiced (Luo *et al.* 2005).

In recent years, there has been a significant prevalence of pomegranate fruit rot symptoms, resembling anthracnose disease in the pomegranate orchards of Golestan province. Therefore, the current research was aimed to characterize the causal agent of the disease by means of morphological and molecular data also evaluate their pathogenicity on pomegranate fruit

Materials and Methods

In summer 2021, progressive anthracnose symptoms on pomegranate fruits in the Gorgan region (Golestan province, Northeast Iran), were observed in several orchards and a total number of 20 pomegranate fruits (collected from 20 orchards) were transferred to the plant pathology laboratory for isolation and pathogen characterization. In order to measure disease incidence, fruits were randomly inspected on 10 pomegranate trees in each garden (10 fruits per tree) and the percentage of symptomatic fruits were calculated. For the isolation purpose, small sections (about 1 × 1 cm) were cut from the margin of symptomatic and healthy tissues and surface-sterilized in a 70% ethanol for 2-3 min and rinsed three times in autoclaved distilled water. Then sections were dried on sterile filter paper, and plated on Potato Dextrose Agar (PDA; Merck, Germany). Plates

were incubated at 25 °C and monitored for fungal growth in two-day intervals. Coelomycetous fungal isolates were recovered from symptomatic tissues, which were initially whitish, turned to dark grey with age, conidiomata sparsely developed on cultures. Pure cultures were established using spore dilution technique; the isolates were preserved in 2 ml micro-tubes tubes containing synthetic nutrient agar (SNA).

Cultural and morphological features were examined on PDA culture medium in dark condition at 25℃, for 14 days. To induce conidiomatal development, the isolates were plated on water agar (WA) with sterilized pine needles and at 25℃ under near-ultraviolet radiation (NUV), with 12/12 h period of time for three weeks. Longitudinal section of conidiomata were mounted in Lactic acid on microscopic slides and examined by an Olympus BX 41 light microscope (Olympus Corporation, Japan). Thirty measurements were made for each of the morphological characters, where possible and 95 % confidence interval were derived, with the extremes given in parentheses. High-resolution photographs of microscopic fungal structures were captured using Olympus digital camera system (DP 25), mounted on light microscope (Olympus Corporation, Japan). Photos were edited using Adobe Photoshop CS6 (Adobe Systems Inc., USA). The isolates were deposited in Culture Collection of Tabriz University (CCTU).

For molecular characterization of the isolates, genomic DNA was extracted from fungal colonies grown on MEA, following the protocol of Möller (Moller *et al.* 1992). Molecular analysis was carried out using sequence data of internal transcribed spacer (ITS) regions and *β-tubulin* gene. ITS-rDNA fragment was amplified using ITS1F/ITS4 primer (White *et al.* 1990). The reaction mixture and thermal cycling conditions were the same as described in Arzanlou *et al.* (2018). The *β-tubulin* gene was amplified using primers T1/Bt2b as described in Mahadevakumar *et al.* (2019). PCR products were visualized on 1% agarose gel stained with ethidium bromide by ball of water-soaked cotton was placed in container electrophoresis in 1X TAE buffer. The amplicons were sequenced in both directions using the same primer set by the Pishgam Biotech Company (Tehran, Iran), Sequence files were edited using SeqMan software in the Laser gene package (DNASTAR Inc., Madison, WI, USA) and consensus sequence was computed using the forward and reverse sequences. The consensus sequence was compared with sequences in the GenBank using the basic local alignment search tool (BLAST). Sequences were deposited in GenBank with the accession numbers PV856197 and PV872039 for ITS and *β-tubulin* gene, respectively. ITS-rDNA and *β-tubulin* gene sequence data for type and reference strain sequences of *Colletotrichum* species were downloaded from GenBank and included in alignment files. To identify the most suitable nucleotide substitution model, MrModelTest version 2.3 was employed. Bayesian phylogenetic analysis was then performed using MrBayes version 3.2.1. Bayesian analyses were accomplished according to previously published procedure (Narmani *et al.* 2019).

Koch's postulates were fulfilled using excised fruit method. Fungal isolates were grown on PDA, and incubated at 25℃ under continuous near fluorescent light seven days before inoculation. Healthy pomegranate fruits sterilized using diluted solution of Javel (50%) for 10 min; 70% ethanol for 1 min, eventually three times washed with sterile distilled water and left to dry under sterile condition. By a sterile scalpel, one cm diameter incision was made on the fruits. A spore dilution was made by washing and filtering seven-day-old PDA culture with autoclaved distilled water and glass wool filter. Initial spore dilution cell-counted by hemacytometer and then sterilized distilled water was added to reach 10^6 spore count per milliliter. Fifty µl of spore dilution was injected on incision section. The wounds were wrapped with Parafilm, to prevent drying. Control fruits were inoculated with sterile distilled water. The inoculated fruits were kept in plastic containers with closed doors and a small maintain relative humidity high (RH>85%). After

two weeks of incubation, the fruits were inspected and the lengths of the lesion and necrosiswas measured from the inoculation point. Re-isolation of the tested isolate was done by cutting small pieces of necrotic tissue from the edge of each lesion and placing on PDA. Fungal isolates were identified as previously described in this paper.

Results and Discussion

The incidence of anthracnose disease in pomegranate orchards of western Golestan province ranged between 20 to 30 percent. Symptoms typically appeared from mid-June to late June, coinciding with increasing temperatures and high humidity levels. The disease was particularly prevalent when temperatures exceed 28°C and relative humidity remained high (90%), creating favorable conditions for fungal development and infection.

Field observations indicated that the most susceptible pomegranate varieties include Malas, Momtaz, and Yousef-Khani, which exhibited severe symptoms under conducive environmental conditions. The disease initially manifests as small, dark brown to black lesions on fruits, which expand and coalesce, leading to significant fruit rot and economic losses.

Figure 1. Pomegranate anthracnose symptoms on the fruit (a-d) Disease symptoms on pomegranate fruit under field conditions.

A total number of 15 isolates were isolated from symptomatic pomegranate fruits samples in Gorgan province. On PDA, fungal colony attained 65 mm diameter in seven days at 25°C in the dark with circular, flat, with dense aerial mycelium and grey to greyish yellow at the center, becoming pale to grey towards the margin. On MEA colonies reached 65 mm in diam. after seven days at 25°C in the dark; circular, flat, with abundant aerial mycelium at the center and with even margins. Colony color whitish at center, grey towards the margin and yellowish to dark grey at reverse side. Acervular conidiomata were produced after three weeks on pine needle surfaces; setae absent. On PDA hyphae 2–9 µm in diameter, smooth-walled, hyaline to pale brown, septate, and branched. Conidial masses were visible around the center and margin, especially with increasing age; setae, sclerotia and chlamydospores were absent. Conidiophores formed directly in acervuli and on mycelia; hyaline, smooth-walled, simple or branched and septate. Conidiogenous cells hyaline, smooth-walled, cylindrical or elongate ampulliform. Conidiogenous cells in aerial mycelia cylindrical, subcylindrical to elongate ampulliform, terminal or intercalary, smooth-walled. Conidia unicellular, smooth, hyaline, mostly with one visible guttules, cylindrical, oblong, obtuse to slightly rounded ends, and 13.5–17.2 × (4.7-)5–5.5(–7.8) µm in size. In slide culture, appressoria mostly formed from mycelia, brown to dark brown, solitary, aseptate, clavate, and other irregular shapes, (4–)5.5–6.5(–9) µm in diam (Figure 1). Based on the morphological characters the isolates were identified as *Colletotrichum gloeosporioides* species complex (Wire *et al.* 2012).

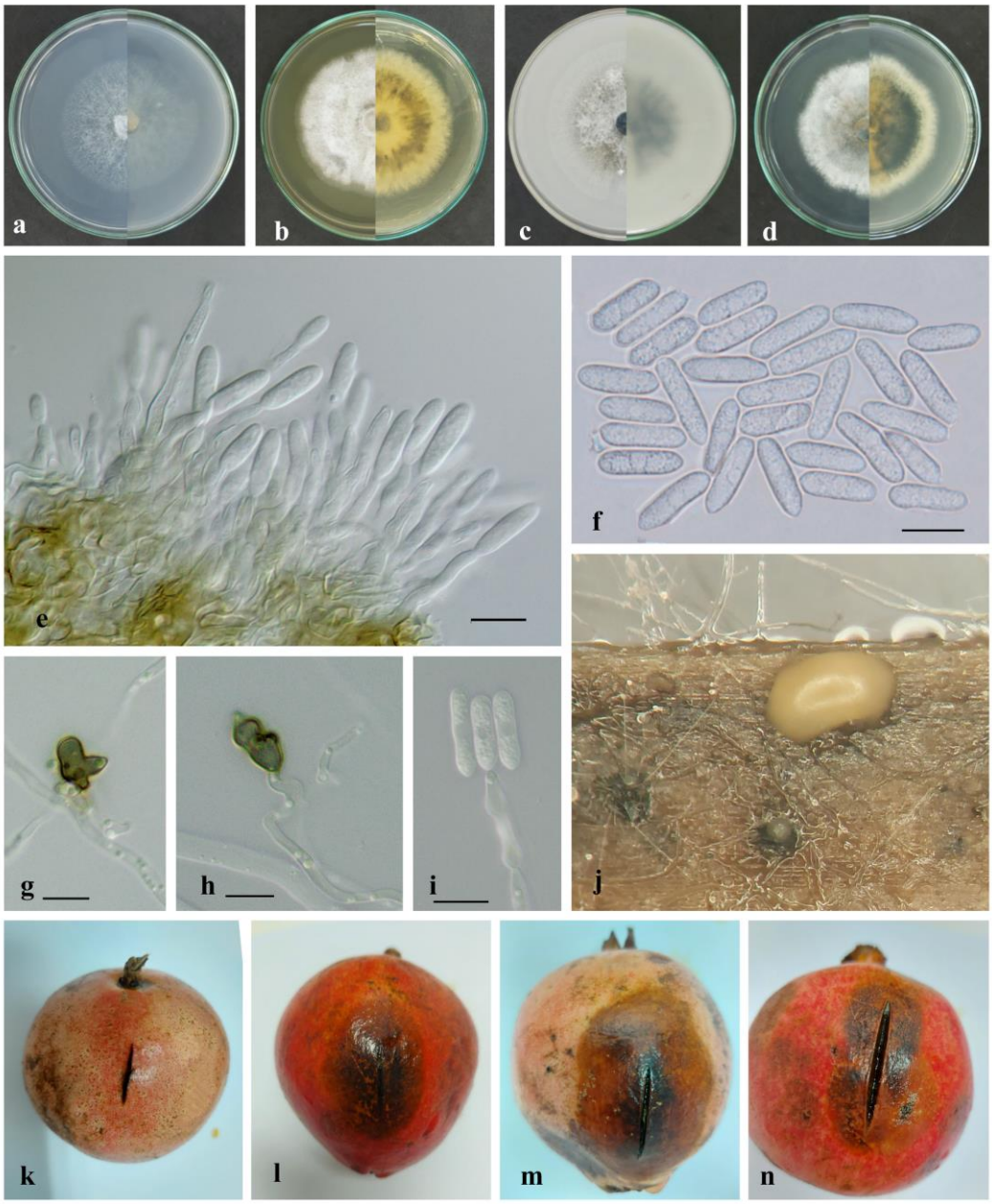

Figure 2. *Colletotrichum theobromicola*. (a-d) Seven day-old colony on SNA, MEA, OA, PDA. (e, i) Conidia and Conidiophores. (f) Conidia. (g, h) Appressoria. (j) Conidiomata formed on pine needles on WA. (k-n) Pathogenicity assay on excised pomegranate fruits after two weeks where k is control.

Morphologically the isolates were identified as member of *C. gloeosporioides* species complex; the identity of species was further confirmed as *C. theobromicola* using sequence data of ITS-rDNA region and *β-tubulin* gene. Megablast search analysis at NCBI's GenBank nucleotide database, based on ITS-rDNA sequence data, showed high similarity with reference sequence of *Colletotrichum* species from GenBank. A phylogeny inferred based on combination of ITS-rDNA sequence and *β-tubulin* gene data obtained in this study together with sequence data from GenBank. The final sequence alignment of the ITS-rDNA sequence and *β-tubulin* gene comprising 61 internal taxa had 840 characters (ITS-rDNA: 1-548, *β-tubulin*: 549-840) and 190 unique site patterns (ITS-rDNA: 74, β-tubulin: 116). *Monilochaetes infuscans* CBS 869.96 was served as the outgroup taxon. Bayesian analyses were performed using the best-fitting substitution

(SYM+I+G for ITS-rDNA and SYM+G for β-tubulin) model and resulted in 2052 generations. After discarding the first 25% of generations as burn-in, the remaining 1540 (75%) generations were used to calculate the consensus Bayesian tree and posterior probabilities. Results indicated that the isolate used in this study resided in *C. theobromicola* clade with highly supported value (Figure 2). Therefore, according to a combination of morphological and phylogenetic data, the isolate was identified as *Colletotrichum theobromicola*.

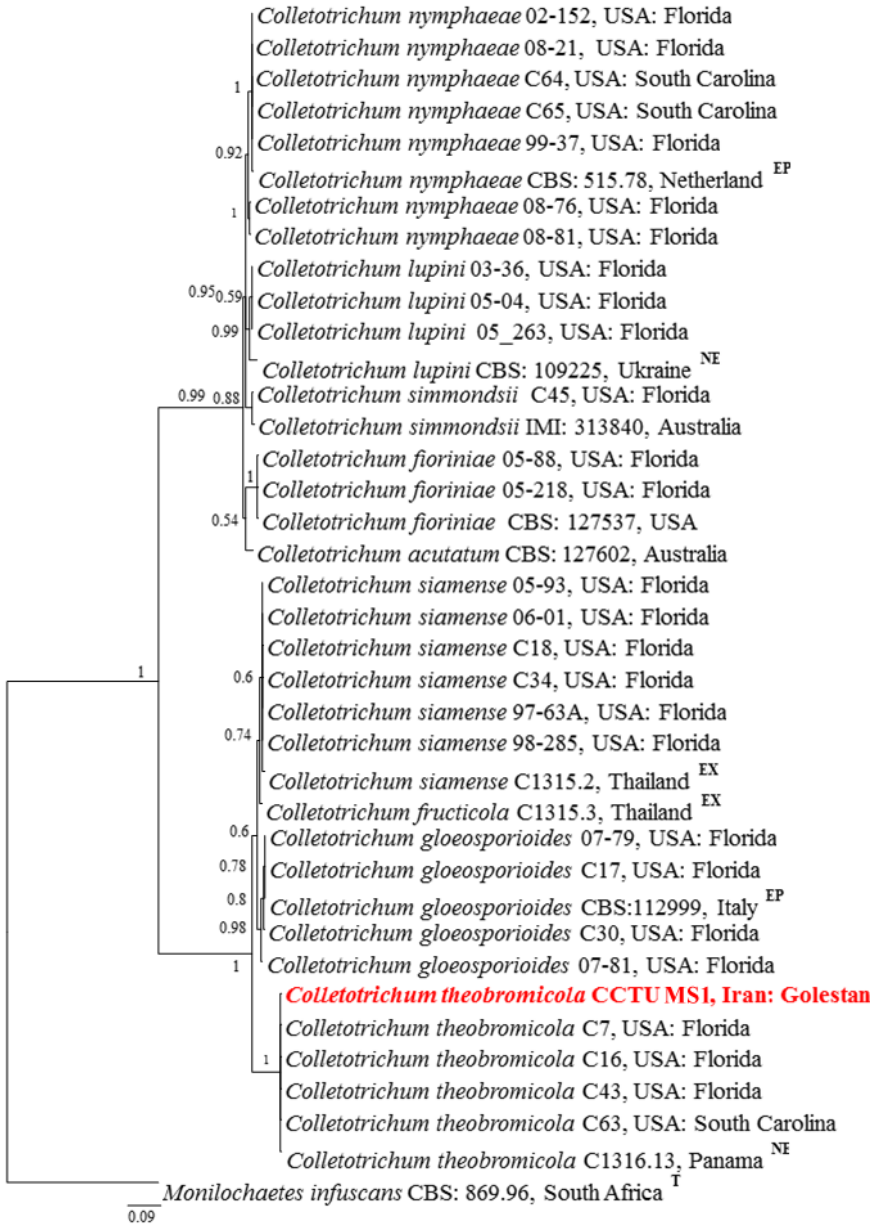

Figure 3. Bayesian inference phylogenetic tree generated using sequences of internal transcribed spacer (ITS rDNA) and *β-tubulin* gene. The representative strain CCTU MS1 in this study is shown in red font. The values above branches show Bayesian posterior probability. The scale bar indicates the number of expected substitutions per site. *Monilochaetes infuscans* (CBS 869.96) was used as the out-group. [EP] = culture from ex-epitype; [EX] = culture from ex-type; [NE] = culture from ex-neotype; [T] = culture from type material.

Colletotrichum theobromicola belongs to *C. gloeosporioides* species complex. Until the present, 37 species have been delineated in this complex, based on multi-gene phylogenetic analyses (Jayawardena *et al.* 2016). Species in this complex can be distinguished only based on multi-

gene sequence phylogenetic analysis. In addition to *C. theobromicola*, several other species in this complex have been reported from pomegranate (Table 1). Besides *C. gloeosporioides* species complex, several species in *C. acutatum* species complex also cause anthracnose fruit rot and leaf blight symptoms on pomegranate worldwide (Table 1).

The results of pathogenicity assay revealed *Colletotrichum theobromicola* being highly pathogenic on excised fruits of Pomegranate. Inoculated fruit of pomegranate began to show symptoms of anthracnose as dark brown to black necrotic lesions surrounding the inoculation point one week after inoculation (Figure 1). The average length of the lesion was 3 cm after two weeks of inoculation; while, no disease symptom was developed on uninoculated controls. Koch's postulates were fulfilled by re-isolation of the fungal isolates from inculcated fruits on PDA. No fungal growth was observed for the controls.

In the present study, C. *theobromicola* isolates were recovered from pomegranate fruits with typical anthracnose symptoms. No disease symptom (leaf spot or blight) was observed on leaves in orchards. However, it has been reported that *C. acutatum* species complex was more aggressive on pomegranate fruits in comparison to *C. gloeosporioides* species complex in artificial inoculation trials (Xavier *et al.* 2019). In contrast, C. *gloeosporioides* species complex has proven more aggressive in leaf inoculation compared to *C. acutatum* species complex (Xavier *et al.* 2019).

Table 1. List of *Colletotrichum* species along with their geographical origin occurring on pomegranate.

	Species	**Organs of pomegranate affected by species**	**Country**	**Reference**
Colletotrichum acutatum species complex				
	C. acutatum sensu stricto	flower anthracnose	Brazil	Belle *et al.* 2018
	C. acutatum sensu stricto	postharvest fruit rot	Italy	Mincuzzi *et al.* 207
	C. fioriniae,	fruit anthracnose and leaf blight	USA	Xavier *et al.* 2019
	C. nymphaeae,	fruit anthracnose and leaf blight	USA	Xavier *et al.* 2019
	C. siamense	fruit anthracnose and leaf blight	USA	Xavier *et al.* 2019
	C. simmondsii	fruit anthracnose and leaf blight	USA	Xavier *et al.* 2019
Colletotrichum gloeosporioides species complex				
	Colletotrichum gloeosporioides	fruit anthracnose and leaf blight	USA	Xavier *et al.* 2019
	C. theobromicola	fruit anthracnose	Australia	Shivas *et al.* 2016
	C. theobromicola	fruit anthracnose	India	Sharma *et al.* 2015
	C. theobromicola	fruit anthracnose and leaf blight	USA	Xavier *et al.* 2019
	C. siamense	fruit anthracnose and leaf blight	USA	Xavier *et al.* 2019
	C. fructicola	fruit anthracnose	China	Hu *et al.* 2023
	C. tropicale	fruit anthracnose	Brazil	Silva-Cabral *et al.* 2019

Colletotrichum theobromicola has proven to occur on a wide host range, representing an economically important pathogen on many important woody hosts (Table 2). This study provides the first report on the occurrence of *C. theobromicola* on pomegranate in Iran; while, it has not been previously reported on any other species from Iran. *Colletotrichum gloeosporioides* has been previously reported as the cause of fruit spot on pomegranate in Iran (Rahimlou *et al.* 2014). There is no other data available on the occurrence and economic impact of *Colletotrichum* spp. on pomegranate in the other regions of Iran. As pomegranate industry is rather big in Iran and pomegranate orchards are distributed throughout the country, there is urgent need to monitor occurrence of *Colletotrichum* spp. on pomegranate orchards in Iran.

Table 2. Host range of *Colletotrichum theobromicola* (Farr & Rossman 2019).

***Colletotrichum theobromicola* (Identified/reported as)**	**Host**	**Country**
Colletotrichum fragariae	*Anacardium occidentale*	Brazil
Colletotrichum fragariae	*Annona cherimola*	Mexico
Colletotrichum fragariae	*Cassia obtusifolia*	USA
Colletotrichum fragariae	*Coffea* sp.	Angola, Brazil
Colletotrichum fragariae	*Cyclamen persicum*	USA
Colletotrichum fragariae	*Fragaria ananassa*	Japan, USA
Colletotrichum fragariae	*Fragaria chiloensis*	USA
Colletotrichum fragariae	*Fragaria* sp.	USA,UK
Colletotrichum fragariae	*Fragaria vesca*	India
Colletotrichum fragariae	*Fragaria virginiana*	India
Colletotrichum fragariae	*Hopea odorata*	Bangladesh
Colletotrichum fragariae	*Potentilla canadensis*	USA
Colletotrichum theobromicola	*Acca sellowiana*	New Zealand
Colletotrichum theobromicola	*Colletotrichum theobromicola*	Australia
Colletotrichum theobromicola	*Allium cepa*	Brazil
Colletotrichum theobromicola	*Allium fistulosum*	Brazil
Colletotrichum theobromicola	*Annona diversifolia*	Mexico
Colletotrichum theobromicola	*Annona muricata*	Brazil
Colletotrichum theobromicola	*Annona squamosa*	Brazil
Colletotrichum theobromicola	*Anthurium* sp.	No country data
Colletotrichum theobromicola	*Butia odorata*	Brazil
Colletotrichum theobromicola	*Buxus microphylla var. japonica*	USA
Colletotrichum theobromicola	*Buxus* sp.	USA
Colletotrichum theobromicola	*Campomanesia phaea*	Brazil
Colletotrichum theobromicola	*Centrosema pubescens*	Thailand
Colletotrichum theobromicola	*Coffea arabica*	Australia, Mexico, Puerto Rico (U.S.A.)
Colletotrichum theobromicola	*Copernicia prunifera*	Brazil
Colletotrichum theobromicola	*Cyclamen persicum*	Israel
Colletotrichum theobromicola	*Eucalyptus ×grandis-urophylla*	Brazil
Colletotrichum theobromicola	*Fragaria ananassa, Fragaria ×ananassa, Fragaria sp., Fragaria vesca*	USA
Colletotrichum theobromicola	*Gossypium indicum*	Korea, Republic of (South Korea)
Colletotrichum theobromicola	*Limonium* sp.	Israel
Colletotrichum theobromicola	*Malpighia emarginata*	Brazil
Colletotrichum theobromicola	*Malus domestica*	USA, Uruguay
Colletotrichum theobromicola	*Mangifera indica*	Colombia, India
Colletotrichum theobromicola	*Manihot esculenta*	Brazil
Colletotrichum theobromicola	*Manilkara zapota*	Brazil
Colletotrichum theobromicola	*Olea europaea*	Argentina, Australia

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پژوهش‌های کاربردی در گیاه‌پزشکی ۱۴ (۱): ۸۱-۷۱ (۱۴۰۴)
مقاله پژوهشی

شناسایی چند منظری و ارزیابی بیماری‌زایی گونه *Colletotrichum theobromicola* روی انار (*Punica granatum*) در ایران

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چکیده

انار با نام علمی *Punica granatum* یک میوه مغذی با خواص متعدد مفید پزشکی و سلامتی می‌باشد. بیماری‌های پوسیدگی میوه یکی از محدودیت های عمده تولید انار در دنیا به شمار می روند که ممکن است در باغ و یا به عنوان بیماری بعد از برداشت در شرایط انبار بروز کنند. بیماری آنتراکنوز که توسط گونه‌های جنس *Colletotrichum* ایجاد می‌شود یکی از بیماری‌های مهم و اقتصادی انار در بسیاری از کشورهای تولید کننده انار به شمار می‌رود، با این وجود اطلاعات در مورد شیوع این بیماری و گونه‌های *Colletotrichum* دخیل در این بیماری در ایران محدود می‌باشد. در سال‌های اخیر علایم مشابه بیماری آنتراکنوز در در باغات انار استان گلستان شیوع قابل توجهی داشته است. بنابراین تحقیق حاضر با هدف شناسایی عامل این بیماری و بررسی بیماری‌زایی قارچ‌های جداسازی شده روی میوه انار اجرا گردید. برای این منظور در سال ۱۴۰۰ نمونه-برداری از باغات انار استان گلستان صورت پذیرفت. جداسازی و خالص سازی عامل بیماری با استفاده از روش‌های رایج در بیماری شناسی گیاهی روی محیط سیب زمینی دکستروز آگار اسیدی صورت گرفت. شناسایی جدایه‌های قارچی استفاده از تلفیق داده‌های توالی نواحی ITS-rDNA و ژن بتا توبولین (beta-tubulin) با داده‌های ریخت شناختی صورت پذیرفت. بر اساس تلفیق داده‌های مولکولی با داده‌های ریخت شناختی، هویت جدایه‌های قارچی (*C. gloeosporioides* species complex) *Colletotrichum theobromicola* تعیین گردید. نتایج آزمون بیماری‌زایی روی میوه انار در شرایط آزمایشگاه نشان‌داد که جدایه‌های قارچی پس از پنج روز باعث بروز علایم پوسیدگی روی میوه انار شدند. با وجود اینکه گونه-های متعددی در دو گونه مرکب *C. gloeosporioides* و*C. acutatum* به عنوان عامل بیماری آنتراکنوز میوه و بلایت برگی انار در دنیا شناسایی شده‌اند، در تحقیق حاضر *C. theobromicola* به عنوان تنها عامل دخیل در ایجاد بیماری آنتراکنوز برای اولین بار در ایران شناسایی گردید. پراکنش جغرافیایی، اهمیت اقتصادی و اکولوژی گونه‌های جنس *Colletotrichum* در مناطق کشت و پرورش انار در ایران نیاز به مطالعه دارند.

کلمات کلیدی: ارزیابی بیماری‌زایی، پوسیدگی میوه، فیلوژنی چندژنی، گونه مرکب *Colletotrichum gloeosporioides*