



Diversity and pathogenicity of *Cladosporium* species associated with alfalfa leaf spot in West Azerbaijan province, Iran

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Abstract

Alfalfa (*Medicago sativa*) is the most important and high-yielding forage legume cultivated worldwide. The aerial parts of the plant play a crucial role in feeding livestock, and the roots contribute to soil nutrient levels, shape microbial communities, and support overall soil improvement. During our field survey of leaf-inhabiting fungi associated with this forage crop, we observed a widespread distribution of cladosporioid leaf spot symptoms in West Azerbaijan province, Iran. However, the causal agents associated with cladosporioid leaf spots and their pathogenicity in northwest Iran remain insufficiently resolved. Accordingly, this study aimed to identify the *Cladosporium* species associated with alfalfa leaf spot and assess their pathogenicity in this region. Samples with leaf spot symptoms were collected from various fields in West Azerbaijan province, including the cities of Miandoab, Mahabad, and Naghadeh, and then subjected to microscopic examination, resulting in the isolation of 49 *Cladosporium* isolates. A three-locus phylogenetic analysis (ITS, *ACT* and *TEF1-α*), combined with a detailed morphological examination of representative isolates, confirmed the association of six *Cladosporium* species with alfalfa leaf spot in Iran: *C. asperulatum*, *C. cladosporioides*, *C. limoniforme*, *C. macrocarpum*, *C. submersum*, and *C. tenellum*. Among these, *C. asperulatum* and *C. submersum* are new records for the fungi of Iran. In addition, *C. asperulatum*, *C. limoniforme*, *C. macrocarpum*, *C. submersum*, and *C. tenellum* are reported for the first time on *M. sativa* in the world. Pathogenicity tests under greenhouse conditions confirmed that four species, including *C. asperulatum*, *C. limoniforme*, *C. macrocarpum*, and *C. submersum*, were pathogenic on alfalfa leaves. These findings clarify the species complex involved in alfalfa leaf spot in northwest Iran and provide an initial evidence base for disease management, with the potential to reduce economic losses.

Keywords Alfalfa · Forage crop · Multigene phylogeny · Taxonomy

Introduction

Alfalfa (*Medicago sativa* L.) is a perennial species in the family *Fabaceae* and an important forage legume crop

worldwide (Al-Snafi et al. 2021). Due to its high nutritional value for livestock, resistance to salinity, ability to improve soil structure and fertility, and capacity to fix nitrogen through symbiosis with rhizobia, it is of particular importance (Meseguer et al. 2021; Tucak et al. 2021). Various biotic and abiotic stresses negatively affect growth and reduce the forage quality and yield of alfalfa. Among biotic stresses, diseases caused by fungi are one of the main factors that limit fodder crop production, leading to reduced yield and quality (Hrbáčková et al. 2020). In alfalfa, foliar leaf spots commonly cause defoliation and loss of photosynthetic leaf area, reducing hay yield and quality (Hwang et al. 2006). Field studies document substantial yield reductions in alfalfa leaf spot complexes

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and, under severe spring black stem epidemics, losses up to 56% (Lan et al. 2024).

The genus *Cladosporium*, first described by Link in 1816, is among the most important dematiaceous hyphomycete genera and is classified under the family *Cladosporiaceae*, order *Cladosporiales*, and class *Dothideomycetes* (Bensch et al. 2012; Bhunjun et al. 2024). *Cladosporium* species exhibit diverse lifestyles, including saprobes, endophytes, and pathogens of plants, animals, and humans (Schubert et al. 2009; Sandoval-Denis et al. 2016; Marin-Felix et al. 2017; Bensch et al. 2018). This genus contains ubiquitous species with global distribution, commonly isolated from diverse sources such as air, soil, plants, food, debris, cloth, paint, and other organic materials (Bensch et al. 2012, 2018; Rosado et al. 2019; Bhunjun et al. 2024). Plant pathogenic species of *Cladosporium* can cause a range of symptoms, including leaf spots, leaf blight, discolorations, necrosis, scab, postharvest rots, and shot hole on stems, leaves, and fruits of various host plants (Bensch et al. 2012; Bhunjun et al. 2024). Different *Cladosporium* species have been reported on *Medicago* species, including *Cladosporium cladosporioides* in China (Zhang 2003), the United States (Morgan-Jones and McKemy 1992), and Poland (Mulencko et al. 2008); *Cladosporium herbarum* in Uzbekistan (Gaponenko 1965); *Cladosporium* sp. in Canada (Ginns 1986) and the United States (French 1989); and *Cladosporium tenuissimum* in China (Han et al. 2019). Notably, *C. tenuissimum* has been confirmed as a causal agent of alfalfa leaf spot in China, reinforcing the contribution of *Cladosporium* species to alfalfa foliar disease burdens (Han et al. 2019). Beyond biomass losses, foliar infection has been shown to depress photosynthetic efficiency and lower crude protein content of alfalfa leaves, directly affecting forage quality (Hwang et al. 2006). Despite these impacts, in Iran there has been no integrative treatment linking species identity with pathogenicity on alfalfa, creating a clear knowledge gap that this study addresses.

The genus *Cladosporium* is morphologically characterized by conidia arranged in acropetal chains, featuring a distinctive coronate scar structure at the conidiogenous loci and conidial hila (scars), consisting of a central convex dome surrounded by a raised periclinal rim (Bensch et al. 2012). Notable morphological features include various types of conidia, such as ramoconidia, secondary ramoconidia, intercalary conidia, and small terminal conidia (Schubert et al. 2007). Through morphological analyses, three major species complexes within this genus have been recognized: *Cladosporium cladosporioides*, *C. herbarum*, and *C. sphaerospermum*

(Schubert et al. 2007; Zalar et al. 2007; Bensch et al. 2012). The majority of *Cladosporium* species can be grouped into one of these three complexes based on their morphological characteristics. However, the existence of numerous cryptic species within these three complexes is well documented (Schubert et al. 2007; Zalar et al. 2007; Bensch et al. 2012, 2018; Sandoval-Denis et al. 2016). Differentiating these *Cladosporium* species is often challenging based solely on morphological traits, as many species share similar characteristics. Therefore, polyphasic approaches, including multi-locus phylogenetic analyses (ITS rDNA, ACT, and TEF1- α), are essential for accurate species delimitation and identification (Marin-Felix et al. 2017). Such approaches are particularly important when establishing etiology for foliar diseases in major crops.

In Iran, West Azerbaijan province alone cultivates ~79,220 ha of alfalfa and produces ~538,221 tonnes annually (Ghaderpour et al. 2018), underscoring the regional importance of safeguarding forage yield and quality. In recent years, leaf spots attributable to *Cladosporium* species have increasingly been observed on *Medicago sativa* in this province, yet the causal species and their pathogenicity remain insufficiently resolved, highlighting the need for targeted investigation. Therefore, the present study aimed to (a) characterize *Cladosporium* species isolated from *M. sativa* in West Azerbaijan province, northwest Iran based on a combination of morphological features and multi-locus phylogenetic analyses and (b) determine the pathogenicity of the identified species under controlled greenhouse conditions to provide a baseline for disease management.

Materials and methods

Sample collection and fungal isolation

Collection of plant material from alfalfa fields showing symptoms of leaf spot caused by *Cladosporium* was carried out from November to December 2020, in the cities of Mian-doab, Mahabad, and Naqadeh in West Azarbaijan province of Iran. Within each field, sampling followed a W-shaped walking pattern; ten evenly spaced stop points were pre-marked along the transect, field edges (≤ 10 m from borders) were avoided to reduce edge effects, and plants were selected at each stop by randomly choosing a direction and taking the third encountered symptomatic plant. From each selected plant, one symptomatic leaflet was chosen at random, and only one lesion per plant was sampled to avoid

pseudoreplication. A total of 76 samples showing different leaf spot symptoms were collected, taken to the laboratory, and examined under a Stemi 305 (Carl Zeiss) stereomicroscope. In order to isolate the fungi, conidia were scraped from a single leaf spot, and single spore cultures were established on malt extract agar (MEA; Merck, Darmstadt, Germany) using a flamed sterilized fine-pointed needle as described by Bakhshi et al. (2021). Out of 49 *Cladosporium* isolates obtained, 13 representative isolates were selected to be used in the phylogenetic analyses and were deposited in the culture collection of the Iranian Research Institute of Plant Protection (IRAN), Tehran, Iran.

DNA extraction and PCR amplification

Genomic DNA was extracted from colonies grown on MEA plates for 10 days at 25 °C in the dark, following the protocol developed by Möller et al. (1992). PCR amplifications were performed in 25 µL reaction mixtures containing 10–15 ng of DNA, 14 µL of *Taq* DNA Polymerase 2x Master Mix RED (Ampliqon, Denmark), and 1 µL of each forward and reverse primers. The amplified and sequenced genomic loci included the internal transcribed spacer (ITS) regions and the intervening 5.8 S nrRNA gene of the nrDNA operon using the primer pair V9G (de Hoog and Gerrits van den Ende, 1998)/ITS4 (White et al. 1990), part of the translation elongation factor 1- α (TEF1- α) gene using the primer pair EF1-728 F/EF1-986R (Carbone and Kohn 1999), and a fragment of the actin (ACT) gene using the primer pair ACT-512 F/ACT-783R (Carbone and Kohn 1999).

The PCR conditions for all loci followed Bakhshi et al. (2022). The final PCR products were examined by electrophoresis on 1% agarose gels containing 0.1 µg/mL ethidium bromide in 1x Tris–Borate–EDTA (TBE) buffer and visualized under UV light to verify amplification purity and size. Sequencing of the amplified PCR products was performed by Microsynth (Balgach, Switzerland).

Multigene phylogenetic analysis

Nucleotide sequence editing was performed using MEGA v. 12 software (Kumar et al. 2024), and then compared with the NCBI's GenBank (www.ncbi.nlm.nih.gov) data using the BLASTn tool. A set of *Cladosporium* sequences and a related genus (outgroup) obtained from GenBank (Supplementary Table 1) were added to our new sequences and aligned with the MAFFT v. 7 online interface using default settings (<http://mafft.cbrc.jp/alignment/software/>) (Rozewicki et al.

2019). The nucleotide substitution models and partition strategies for each locus were calculated by MrModeltest v. 2.3 (Nylander 2004) based on the Akaike Information Criterion (AIC). The alignments of individual loci were concatenated with Mesquite v. 3.8.1 (Maddison and Maddison, 2023). Bayesian Inference (BI) analyses using a concatenated matrix of ITS, *ACT* and *TEF1- α* loci were executed using MrBayes v. 3.2.6 (Ronquist et al. 2012). The heating parameter was set at 0.15, and the Markov chain Monte Carlo (MCMC) analysis of four chains was started in parallel from a random tree topology and lasted until the average standard deviation of split frequencies fell below 0.01 (stop value). Trees were saved every 1,000 generations and the first 25% of saved trees were discarded as the burn-in phase and posterior probabilities (PP) were determined from the remaining trees. The resulting phylogenetic trees were visualized with Geneious v. 8.1.8 (Kearse et al. 2012) and edited in Adobe® Illustrator 2024 Artwork v. 28.0. The new sequences were deposited in GenBank (www.ncbi.nlm.nih.gov) (Table 1).

Table 1 Genbank accession numbers of *Cladosporium* spp. Isolates obtained from alfalfa-producing regions in this study

Species	Isolates	GenBank accession numbers		
		ITS	actA	tef1
<i>Cladosporium asperulatum</i>	IRAN 4656 C	PX369115	PX379573	PX379586
<i>Cladosporium cladosporioides</i>	IRAN 4655 C		PX379574	
<i>Cladosporium cladosporioides</i>	IRAN 4663 C		PX379575	
<i>Cladosporium limoniforme</i>	IRAN 4658 C	PX369116	PX379576	PX379587
<i>Cladosporium macrocarpum</i>	IRAN 4654 C		PX379577	
<i>Cladosporium macrocarpum</i>	IRAN 4657 C		PX379578	
<i>Cladosporium macrocarpum</i>	IRAN 4660 C		PX379579	PX379588
<i>Cladosporium macrocarpum</i>	IRAN 4661 C		PX379580	
<i>Cladosporium macrocarpum</i>	IRAN 4662 C		PX379581	
<i>Cladosporium submersum</i>	IRAN 4659 C	PX369117	PX379582	
<i>Cladosporium tenellum</i>	IRAN 4669 C		PX379583	PX379589
<i>Cladosporium tenellum</i>	IRAN 4670 C	PX369118	PX379584	
<i>Cladosporium tenellum</i>	E9		PX379585	PX379590

Morphological characterization

Microscopic features of isolates were studied on synthetic nutrient-poor agar (SNA; Crous et al. 2019) after incubation at 25 °C for 7–15 days. Slide preparations were mounted in clear lactic acid and/or in Shear's solution using the inclined coverslip method (Nugent et al. 2006) and also transparent adhesive tape (Bensch et al. 2012). For each isolate, 30 measurements were made at 1000× magnification using a BX51 (Olympus) light microscope for each microscopic structure, and 95% confidence intervals were derived for the measurements with extreme values given in parentheses. The terminology of morphological structures followed those used by (Bensch et al. 2012; El-Dawy et al. 2021). High-resolution photographic images of microscopic fungal structures were captured with an Olympus DP25 camera attached to the above-mentioned light microscope. Adobe Photoshop PS2023 was used for the final editing of acquired images and photographic preparations.

For cultural characteristics, isolates were cultivated on MEA, potato dextrose agar (PDA; Merck, Darmstadt, Germany), and SNA for 15 days at 25 °C in darkness, after which the surface and reverse colors of the colonies were rated using the mycological color charts of Rayner (1970).

Pathogenicity tests

To confirm the pathogenicity, representative isolates of the identified species were evaluated by inoculating alfalfa plants in the greenhouse. Plastic pots were filled with a mixture of field soil, perlite, and sand (1:2:1 ratio), which had been autoclaved three times, and alfalfa seeds were planted and incubated at 25–27 °C for ten days, in the greenhouse. Pots were randomly assigned to treatments in a completely randomized design (CRD) with pots as the experimental unit; three pots (replicates) were used per isolate. The entire experiment was conducted twice in independent runs, and bench positions were rotated during incubation to minimize positional effects. For the pathogenicity test, conidial suspensions of the representative isolates were collected from 2-week-old colonies grown on MEA (in six cm plates) at 25 °C and homogenized in sterile distilled water. The suspensions were filtered through two layers of sterile cheesecloth, diluted in distilled water with 0.05% (v/v) Tween 20, and adjusted to 1×10^6 conidia/mL using a hemocytometer (Han et al. 2019; Hu 2023; Gu et al. 2024). The suspensions were sprayed on the three-week-old plants. No artificial wounding or mechanical damage was introduced to the

leaf surfaces to ensure noninvasive inoculation conditions. Three control pots of alfalfa plants were sprayed with sterile distilled water. The plants were individually covered with plastic bags to maintain a relative humidity of 100% for 48 h, after which the pots were kept in a greenhouse with 80% humidity at 28 ± 2 °C for three weeks. Evaluations were performed daily to monitor the appearance of symptoms. Because the study objective was etiological confirmation, pathogenicity was scored qualitatively as leaf spot present (1) or absent (0) at the pot level. To fulfill Koch's postulates, all isolates were re-identified morphologically based on the structures produced on the leaf spots.

Results

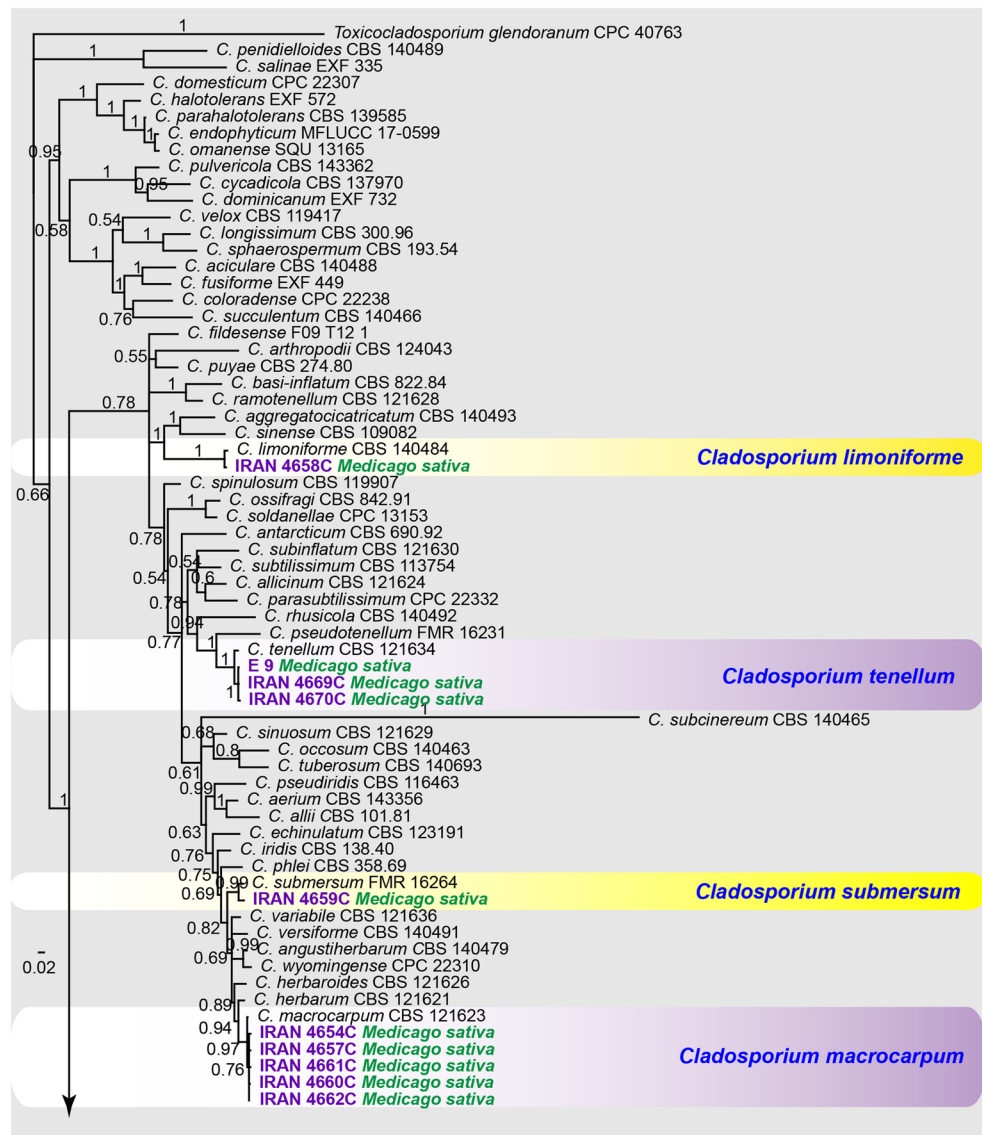
Phylogeny

The concatenated alignment included 157 ingroup taxa within the genus *Cladosporium* (144 taxa from NCBI and 13 taxa from this study) (Table 1) with 2,410 characters including alignment gaps, divided into three partitions. The genomic loci boundaries were as follows: 1–644 characters for ITS, 645–912 characters for *ACT*, and 913–2410 characters for *TEF-1 α* . *Toxicocladosporium glendoranum* Crous & Jurjević (isolate CPC 40763) was used as the outgroup. According to the results from the model testing software, the GTR+I+G evolution model was applied to all three loci, with Dirichlet (1, 1, 1, 1) base frequencies. Among the 2,410 characters used for Bayesian inference, 1,224 unique spatial patterns were identified, comprising 210, 241, and 773 nucleotides corresponding to the ITS, *ACT*, and *TEF-1 α* genomic regions, respectively. Bayesian analysis resulted in 3,590,000 generations and 7,182 trees. The 1,794 trees (25%) with the lowest probability values were discarded, and the probability values were determined from the consensus tree constructed from the remaining 5,388 trees (75%) (Fig. 1).

Taxonomy

The Consolidated Species Concept was applied in this investigation to identify species, revealing that the 13 isolates could be assigned to six *Cladosporium* species: *C. asperulatum*, *C. cladosporioides*, *C. limoniforme*, *C. macrocarpum*, *C. submersum*, and *C. tenellum*. The species are treated as follows:

Fig. 1 Phylogenetic tree inferred by Bayesian analysis of the combined three-locus (ITS, *tefl* and *actA*) sequence alignment using MrBayes v. 3.2.6 for *Cladosporium* spp. The scale bar indicates 0.02 expected changes per site. Species of this study are indicated in coloured blocks and isolates from this study are highlighted in bold



Cladosporium asperulatum Bensch, Crous & U. Braun (Fig. 2).

Description in vitro: on SNA: *Mycelium* consisting of superficial, immersed, septate, subhyaline to pale brown, branched hyphae, 3 µm wide. *Conidiophores* arise laterally and terminally from the hyphae, erect or slightly flexuous, septate, unbranched, pale brown to brown, smooth, verruculose, or slightly rough, 55–100 × 3–4 µm. *Conidiogenous cells* are integrated, terminal or intercalary, geniculate and nodulose, (25–)29–36(–55) × 3–4 µm, with sympodial proliferation and up to five apical loci. *Ramoconidia* cylindrical to subcylindrical, pale brown, smooth, minutely verruculose, or slightly rough, 0(–1)-septate, sometimes constricted at the septum, (10–)14–18(–28) × 3 µm. *Conidia* formed

in short branched chains, fusiform to ellipsoidal or subcylindrical in shape, pale brown, smooth to slightly verruculose, aseptate, with protuberant, thickened, and darkened hila, intercalary conidia (7–)10–12(–17) × 3 µm, terminal conidia (3–)5–7(–10) × (1–)2–3 µm.

Specimens examined. *Medicago sativa*: IRAN, West Azerbaijan province, Miandoab, Shabanli, 37°07'40"N, 46°01'11"E, 11 Nov. 2020, M. Kheiri-Ghaleh (IRAN 4656 C).

Cladosporium cladosporioides (Fresen.) G.A. de Vries (Fig. 3).

Description and illustration: Bensch et al. (2018)

Specimens examined. *Medicago sativa*: IRAN, West Azerbaijan province, Miandoab, Davachi, 37°05'24"N,

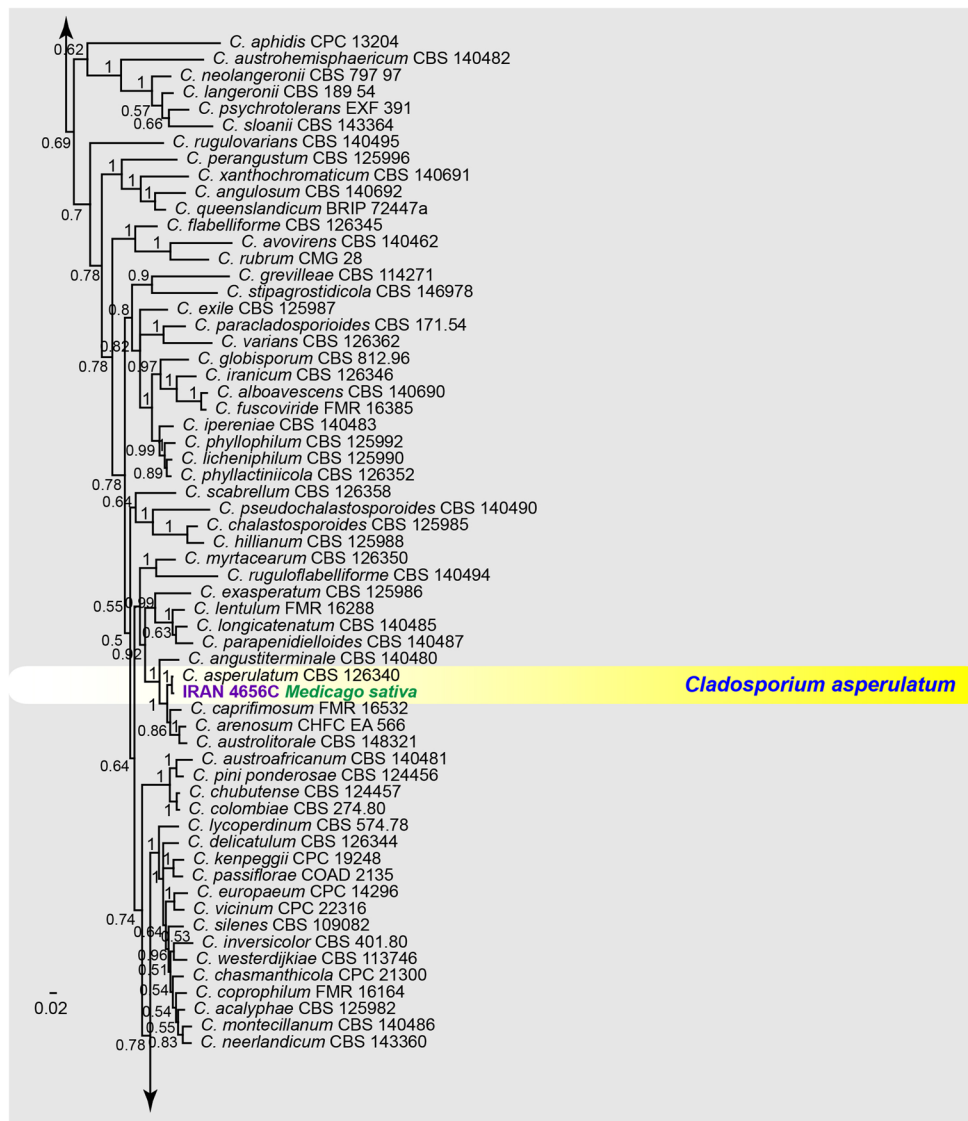


Fig. 1 (continued)

46°03'09"E, 11 Nov. 2020, M. Kheiri-Ghaleh (IRAN 4655 C); Miandoab, Baktash, 37°04'31"N, 46°00'02"E, 11 Nov. 2020, M. Kheiri-Ghaleh (IRAN 4663 C).

Cladosporium limoniforme Bensch, Crous & U. Braun (Fig. 4).

Description in vitro: on SNA: *Mycelium* consisting of superficial, immersed, septate, sparse, usually unbranched, pale olivaceous brown, 2 µm wide, minutely verruculose hyphae. *Conidiophores* are dimorphic, short and may only appear as very short lateral branches of hyphae. *Conidiophores* micronematous to macronematous, subhyaline to pale olivaceous brown, smooth or slightly rough-walled

surface, unbranched, rarely branched, and usually neither geniculate nor nodulose, sometimes hardly distinguishable from hyphae, usually reduced to conidiogenous cells, 60–100 × 3 µm. *Conidiogenous cells* integrated, terminal, occasionally intercalary, and narrowly cylindrical, neither geniculate nor nodulose, (25–)35–42(–50) × 3 µm. *Ramiconidia* aseptate, rarely one septate, (10–)13–15(–21) × 4 µm. *Conidia* catenate, in short branches, pale olivaceous brown or pale brown, limoniform, obovoid to ellipsoid, with rounded apex and attenuated base, aseptate, with protuberant and thickened hila, intercalary conidia 3–4(–6) × 3 µm, terminal conidia 5–6(–8) × 3–3(–4) µm.

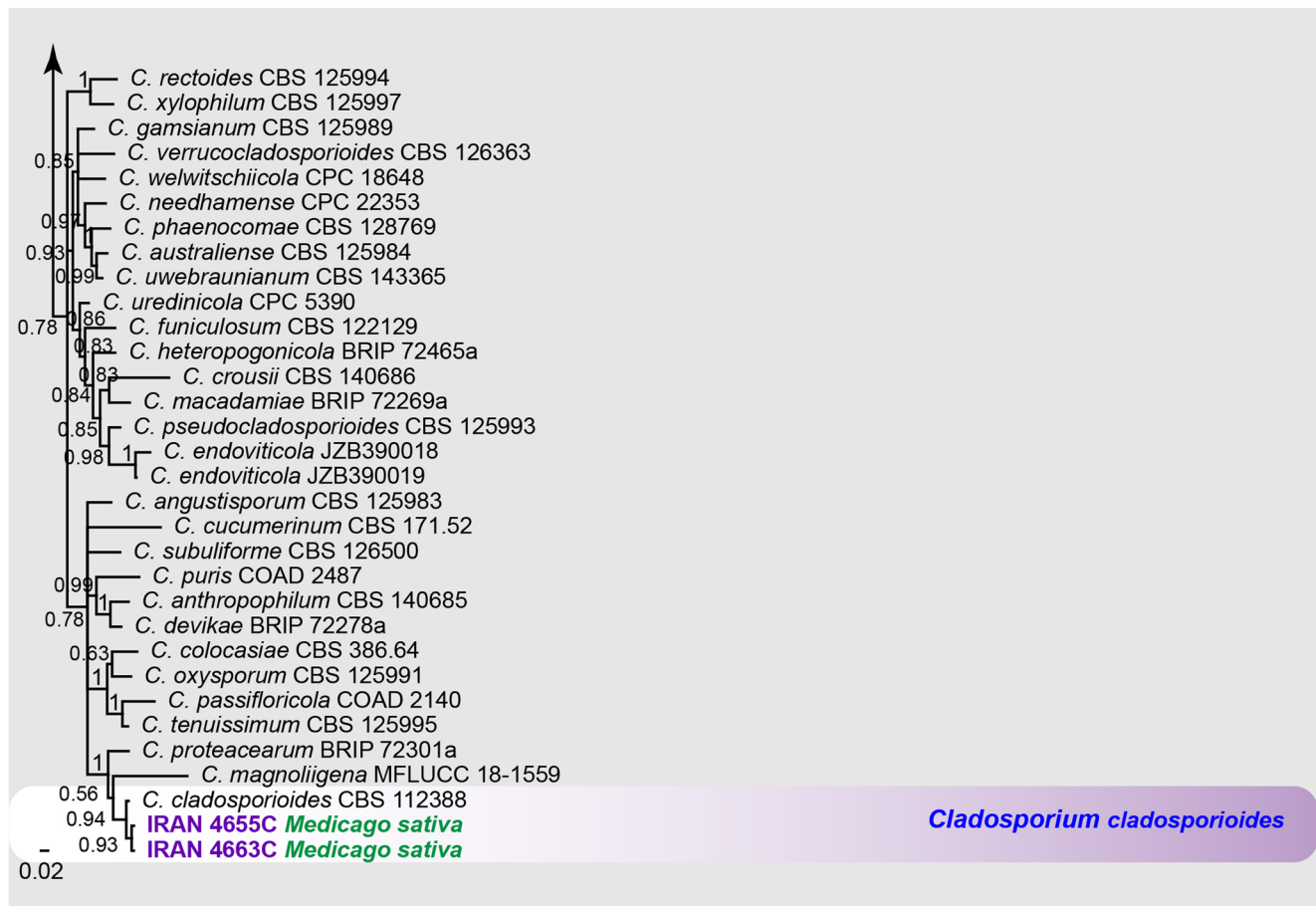


Fig. 1 (continued)

Specimens examined. *Medicago sativa*: IRAN, West Azerbaijan province, Miandoab, Jarchiloo, 37°04'28"N, 46°03'13"E, 11 Nov. 2020, M. Kheiri-Ghaleh (IRAN 4658 C).

Cladosporium macrocarpum Preuss (Fig. 5).

Description and illustration: Bensch et al. (2012, 2018)

Specimens examined. *Medicago sativa*: IRAN, West Azerbaijan province, Miandoab, Baktash, 37°07'19"N, 46°00'50"E, 11 Nov. 2020, M. Kheiri-Ghaleh (IRAN 4657 C); Miandoab, Khazine, 37°07'27"N, 46°02'50"E, 11 Nov. 2020, M. Kheiri-Ghaleh (IRAN 4654 C); Miandoab, Baktash, 37°04'31"N, 46°00'02"E, 12 Nov. 2020, M. Kheiri-Ghaleh (IRAN 4660 C); Miandoab, Shabanli, 37°07'39"N, 46°01'23"E, 11 Nov. 2020, M. Kheiri-Ghaleh (IRAN 4661 C); Miandoab, Davachi, 37°05'24"N, 46°03'09"E, 12 Nov. 2020, M. Kheiri-Ghaleh (IRAN 4662 C).

Cladosporium submersum Iturrieta-González, Dania García & Gené (Fig. 6).

Description in vitro: on SNA: *Mycelium* consisting of superficial, septate, branched subhyaline to pale brown, smooth walls to verruculose, 3 µm wide hyphae. *Conidiophores* micronematous or macronematous, arising laterally and terminally from hyphae, unbranched, erect to slightly flexuous, geniculate at the apex, pale brown to brown, smooth to verruculose, usually reduced to conidiogenous cells, (35–)69–78(–82) × 3–5 µm. Conidiogenous cells integrated, intercalary or terminal, geniculate-sinuous, (12–)23–28(–33) × 3–5 µm. *Ramiconidia* rarely formed, aseptate, rarely one septate, cylindrical to subcylindrical, (14–)17–19(–23) × 4–5(–6.5) µm. *Conidia* catenate, in short branched chains, pale brown, verrucose, ovoid to ellipsoidal or subcylindrical, with rounded apex and attenuated base, aseptate, with protuberant and slightly darkened hila, intercalary conidia (8–)12–15(–22) × 4–5(–7) µm, terminal conidia (6–)7–8(–13) × 4–5 µm.

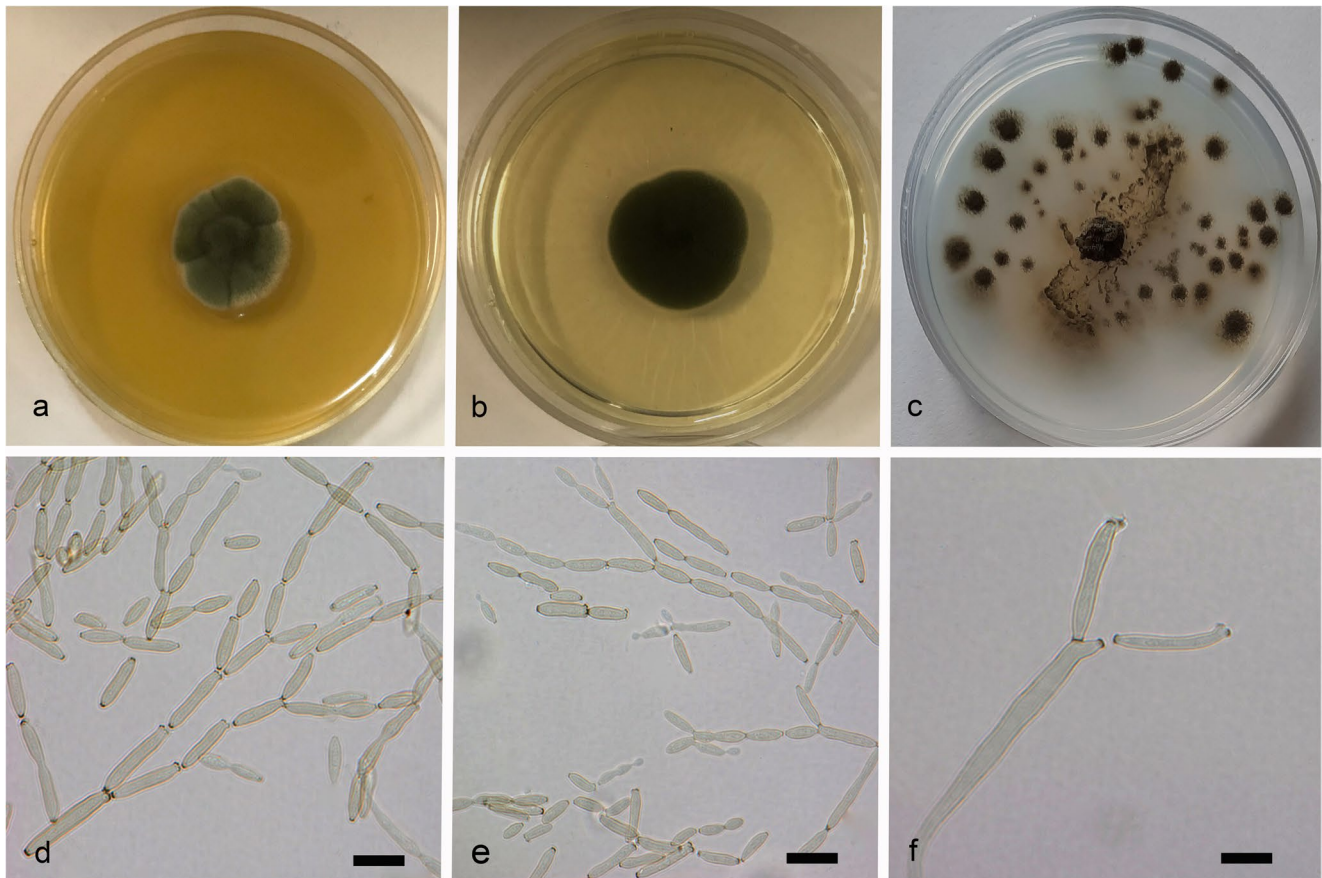


Fig. 2 *Cladosporium asperulatum* (IRAN 4656 C). **a** Culture on MEA. **b** Culture on PDA. **c** Culture on SNA. **d–f** Conidiophores, ramoconidia and conidia. Scale bars = 10 μ m

Specimens examined. *Medicago sativa*: IRAN, West Azerbaijan province, Miandoab, Baktash, 37°07'09"N, 46°00'42"E, 11 Nov. 2020, M. Kheiri-Ghaleh (IRAN 4659 C).

Cladosporium tenellum K. Schub., Zalar, Crous & U. Braun (Fig. 7).

Description and illustration: Bensch et al. (2012, 2018).

Specimens examined. *Medicago sativa*: IRAN, West Azerbaijan province, Miandoab, Shabanli, 37°07'40"N 46°01'11"E, 11 Nov. 2020, M. Kheiri-Ghaleh (IRAN 4669 C). Miandoab, Baktash, 37°04'31"N 46°00'02"E, 12 Nov. 2020, M. Kheiri-Ghaleh (IRAN 4670 C). Miandoab, Shabanli, 37°07'39"N 46°01'23"E, 11 Nov. 2020, M. Kheiri-Ghaleh (E 9).

Pathogenicity tests

The results of the pathogenicity test for *Cladosporium* species revealed that, out of the six selected isolates representing six *Cladosporium* species, four species including *C. asperulatum* (IRAN 4656 C), *C. limoniforme* (IRAN 4658 C), *C. macrocarpum* (IRAN 4657 C), and *C. submersum* (IRAN 4659 C), exhibited positive pathogenicity, causing leaf spot symptoms on the inoculated alfalfa leaves (Fig. 8). The control alfalfa plants, inoculated with sterile water, did not develop any lesions. Pathogenicity tests for *C. cladosporioides* (IRAN 4655 C) and *C. tenellum* (IRAN 4669 C)

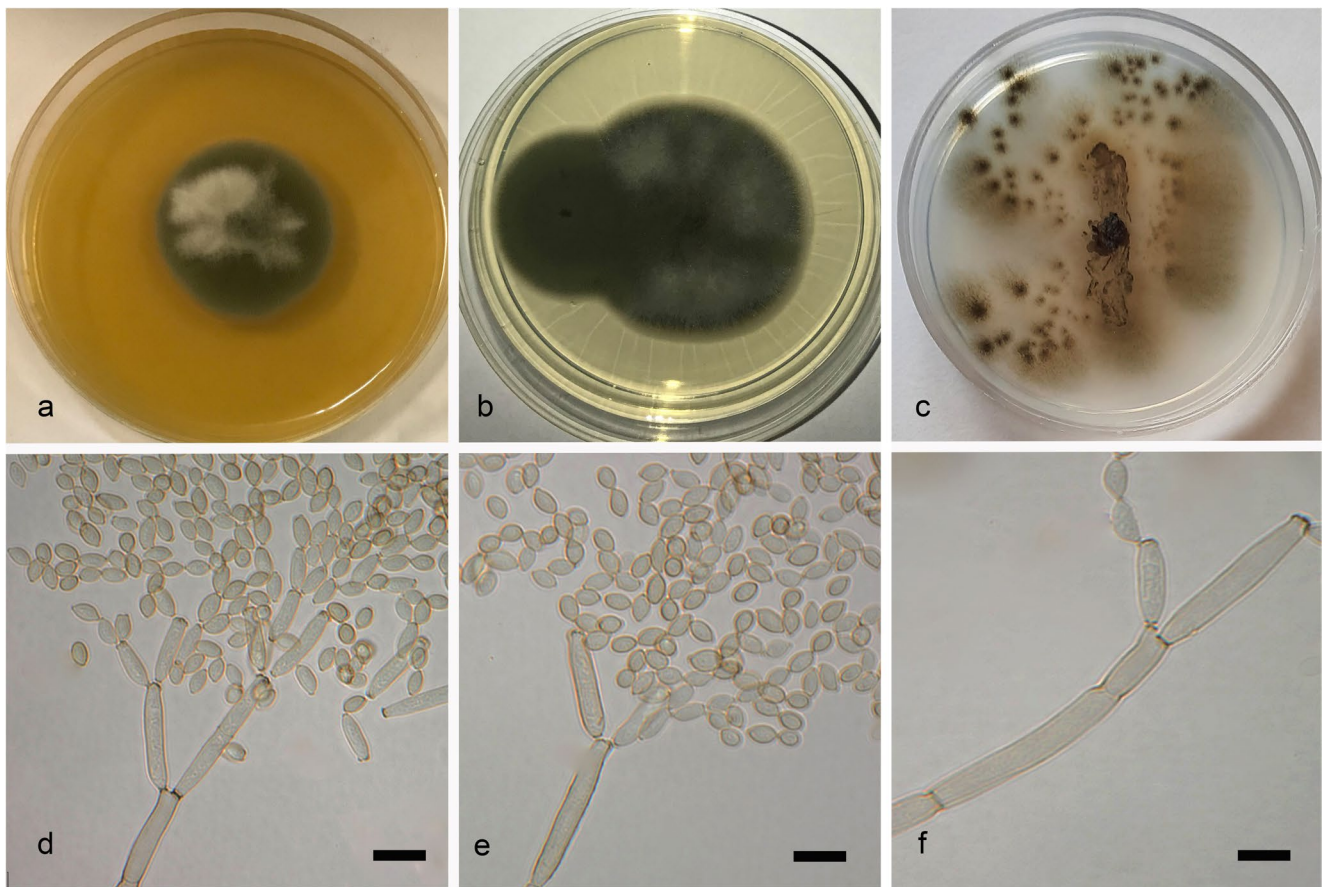


Fig. 3 *Cladosporium cladosporioides* (IRAN 4655 C). **a** Culture on MEA. **b** Culture on PDA. **c** Culture on SNA. **d–f** Conidiophores, ramoconidia and conidia. Scale bars = 10 µm

yielded negative results. The symptoms typically appeared as necrotic spots on plant leaves, beginning at the leaf edges. The spots were generally white to brown with darkened borders. The pathogenicity of the species was confirmed by reisolating the inoculated fungi from the resulting lesions.

Discussion

Alfalfa (*Medicago sativa*) is recognized globally as a key forage legume due to its high nutritional value and contribution to livestock feeding (Meseguer et al. 2021; Tucak et al. 2021). The significance of its aerial and root

systems extends beyond mere productivity; alfalfa plays a pivotal role in soil health, impacting nutrient cycling and fostering beneficial microbial communities (Tucker et al. 2024). However, its production is often threatened by fungal pathogens that cause leaf spot diseases, leading to reduced photosynthetic capacity and yield losses. In this context, we investigated cladosporioid leaf spots on alfalfa in West Azerbaijan (Iran) using an integrated approach. This research employed an integrated species concept, combining morphological and phylogenetic approaches, to facilitate accurate identification of *Cladosporium* species associated with alfalfa in Iran. In addition, greenhouse pathogenicity tests (designed to fulfill

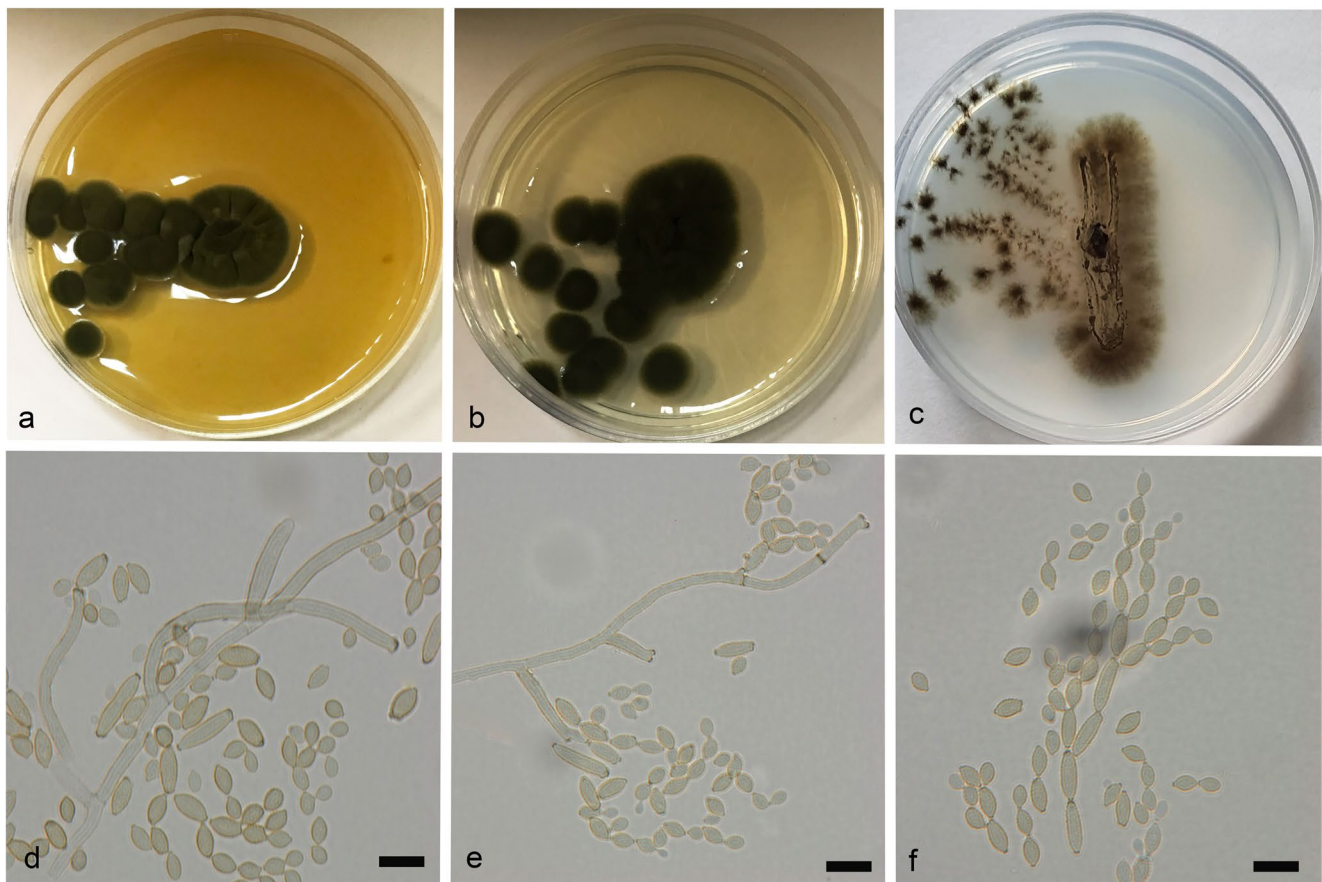


Fig. 4 *Cladosporium limoniforme* (IRAN 4658 C). **a** Culture on MEA. **b** Culture on PDA. **c** Culture on SNA. **d–f** Conidiophores, ramoconidia and conidia. Scale bars = 10 μ m

Koch's postulates) were conducted, with qualitative presence/absence scoring at the pot level and independent experimental repeats, assessing the potential impacts of these *Cladosporium* species on alfalfa cultivation. Together, these complementary approaches allow both precise species delimitation in cryptic complexes and an etiological link to disease symptoms, which is essential for developing management strategies.

The widespread distribution of leaf spot symptoms observed during our field survey indicates that *Cladosporium* species pose a significant threat to alfalfa cultivation within the West Azerbaijan province of Iran. Sequences

of three nuclear loci (ITS, *ACT*, and *TEF1- α*) revealed an unexpected species diversity and highlighted the prevalence of leaf spot diseases caused by *Cladosporium* species in alfalfa crops in this region. The identification of six *Cladosporium* species—*C. asperulatum*, *C. cladosporioides*, *C. limoniforme*, *C. macrocarpum*, *C. submersum*, and *C. tenellum*—associated with alfalfa leaf spot disease is particularly noteworthy. Among these, the discovery of *C. asperulatum* and *C. submersum* as new records for the fungi of Iran, emphasizes the need for further research into the fungal diversity associated with local crops. Additionally, the first world reports of *C. asperulatum*,

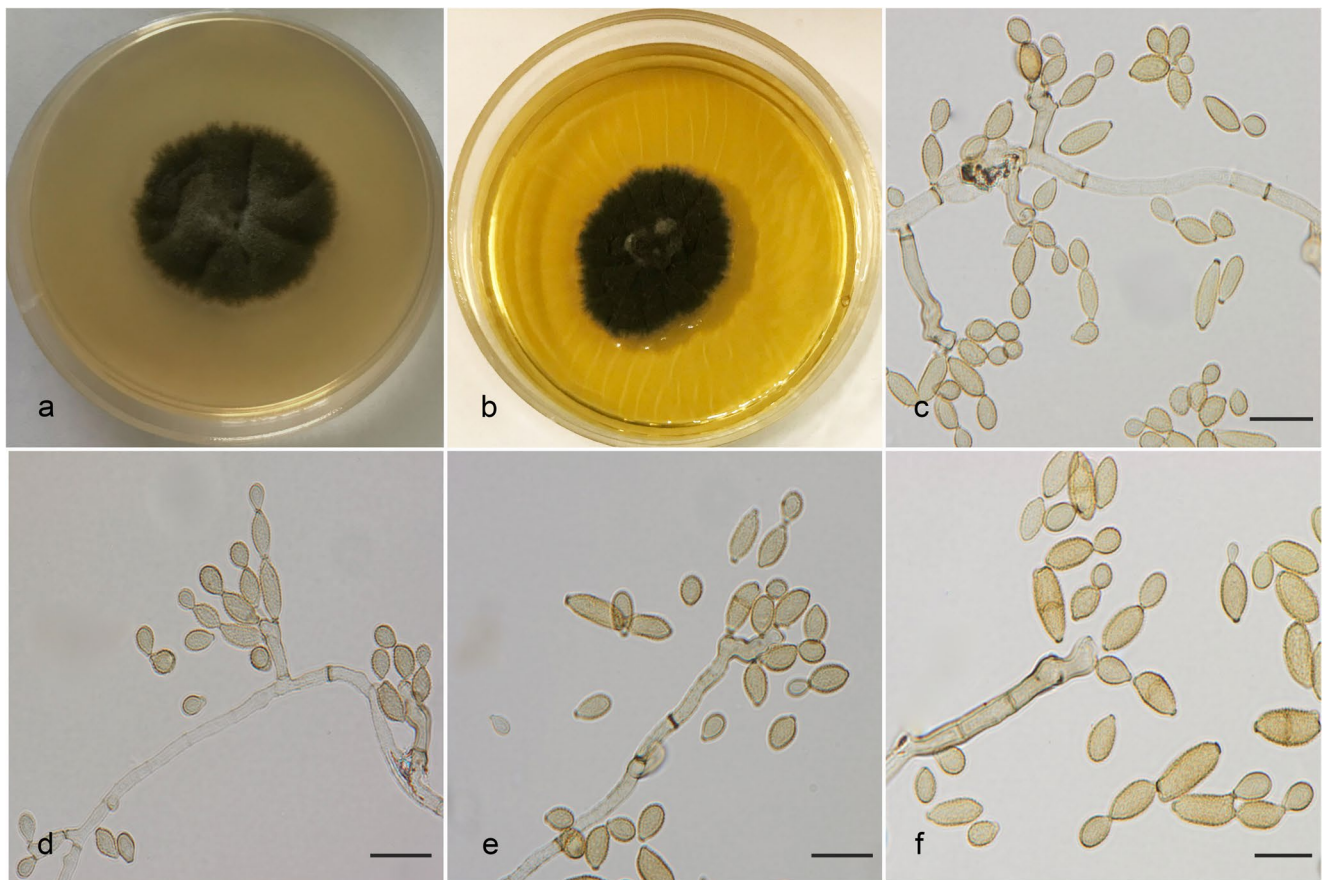


Fig. 5 *Cladosporium macrocarpum* (IRAN 4661 C). **a** Culture on MEA. **b** Culture on PDA. **c–f** Conidiophores, ramoconidia and conidia. Scale bars = 10 μ m

C. limoniforme, *C. macrocarpum*, *C. submersum*, and *C. tenellum* on *M. sativa* highlight the expanding understanding of fungal associations with this important forage crop. Comparable multi-species assemblages of *Cladosporium* have also been documented on other hosts (e.g., citrus), underscoring both the ecological breadth of the genus and the likelihood of poly-etiological leaf spot complexes in the field (Zheng et al. 2025).

The morphological characteristics of the *Cladosporium* species recorded in the present study were consistent with those reported in other investigations under similar conditions (Bensch et al. 2018; Iturrieta-González et al.

2021). However, the six *Cladosporium* species recognized in this study are almost indistinguishable morphologically and require multilocus phylogeny for species-level resolution. This accords with the recognized presence of cryptic taxa within the *C. cladosporioides* and allied complexes (Bensch et al. 2012). Accordingly, accurate species-level identification in this pathosystem should rely on multilocus phylogeny complemented by careful morphological assessment.

We also assessed the pathogenicity of the obtained *Cladosporium* species. Under greenhouse conditions, pathogenicity tests indicated that *C. asperulatum*, *C.*

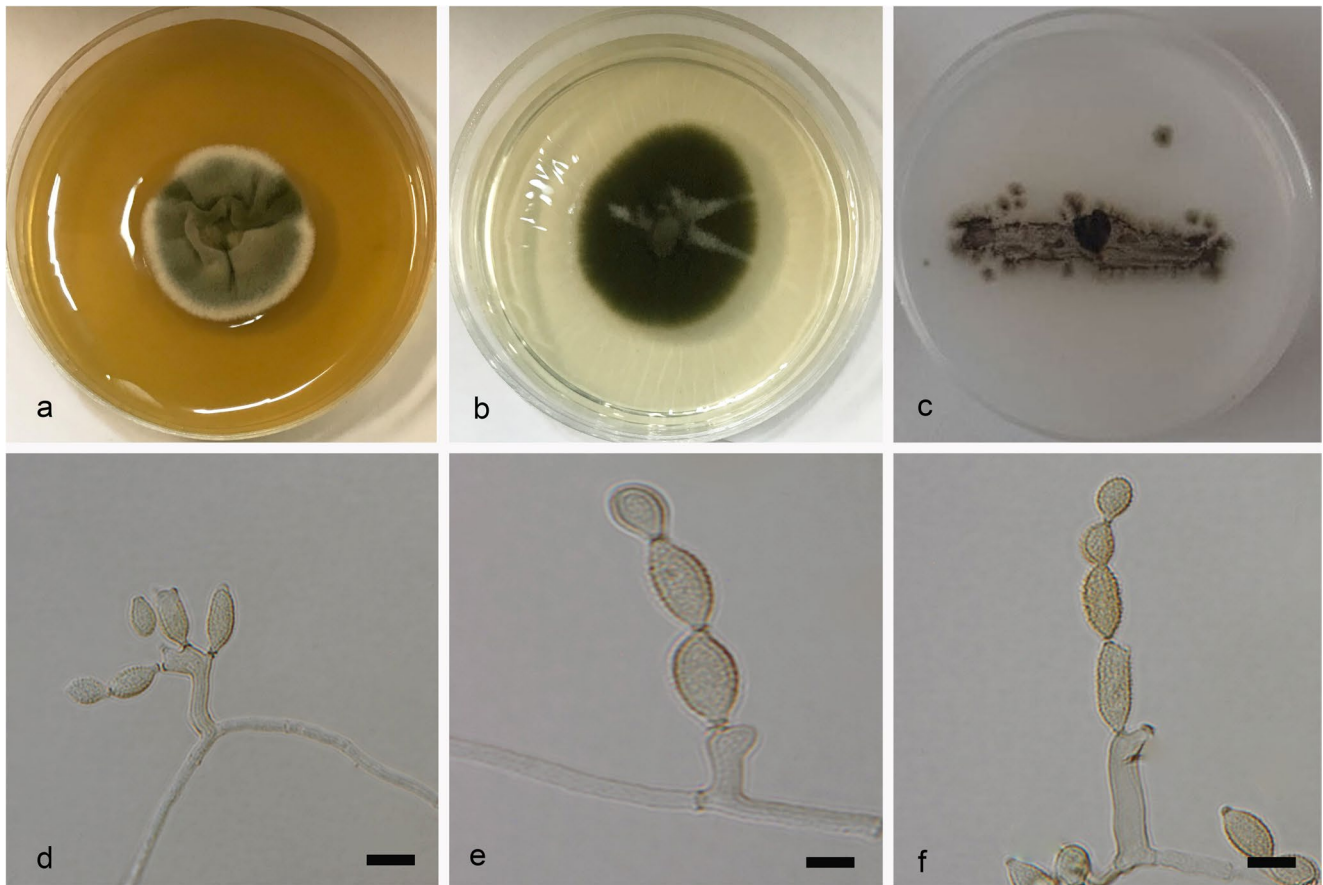


Fig. 6 *Cladosporium submersum* (IRAN 4659 C). **a** Culture on MEA. **b** Culture on PDA. **c** Culture on SNA. **d–f** Conidiophores, ramoconidia and conidia. Scale bars=10 µm

limoniforme, *C. macrocarpum*, and *C. submersum* were pathogenic to alfalfa plants. In contrast, *C. cladosporioides* and *C. tenellum* did not induce symptoms. This result may be explained by their endophytic lifestyle, weak virulence, or host-specific interactions, as has also been reported in other studies (Schubert et al. 2007; Sandoval-Denis et al. 2016). Multiple studies highlight that species within the *C. cladosporioides* complex span lifestyles from endophytes and saprobes to pathogens, and even include strains developed as biocontrol agents; thus, non-pathogenic outcomes in greenhouse tests are not unexpected for some isolates (Iturrieta-González et al. 2021; Lee et al. 2023; Yang et al. 2023; Torres

et al. 2017). For example, *C. cladosporioides* has been explored as an antagonist of foliar pathogens (e.g., *Venturia inaequalis*, *Puccinia horiana*) (Köhl et al. 2015; Torres et al. 2017), whereas pathogenic populations have been reported on other crops such as citrus, tomato and rubber, indicating context-dependent virulence and host range (Zheng et al. 2025; Robles-Yerena et al. 2019; Gomes et al. 2023).

Based on our results, the *Cladosporium* isolates associated with cladosporioid leaf spot on alfalfa in Iran comprise a multi-species complex rather than a single etiological agent. The coexistence of multiple *Cladosporium* species on the same host suggests that mixed infections may occur

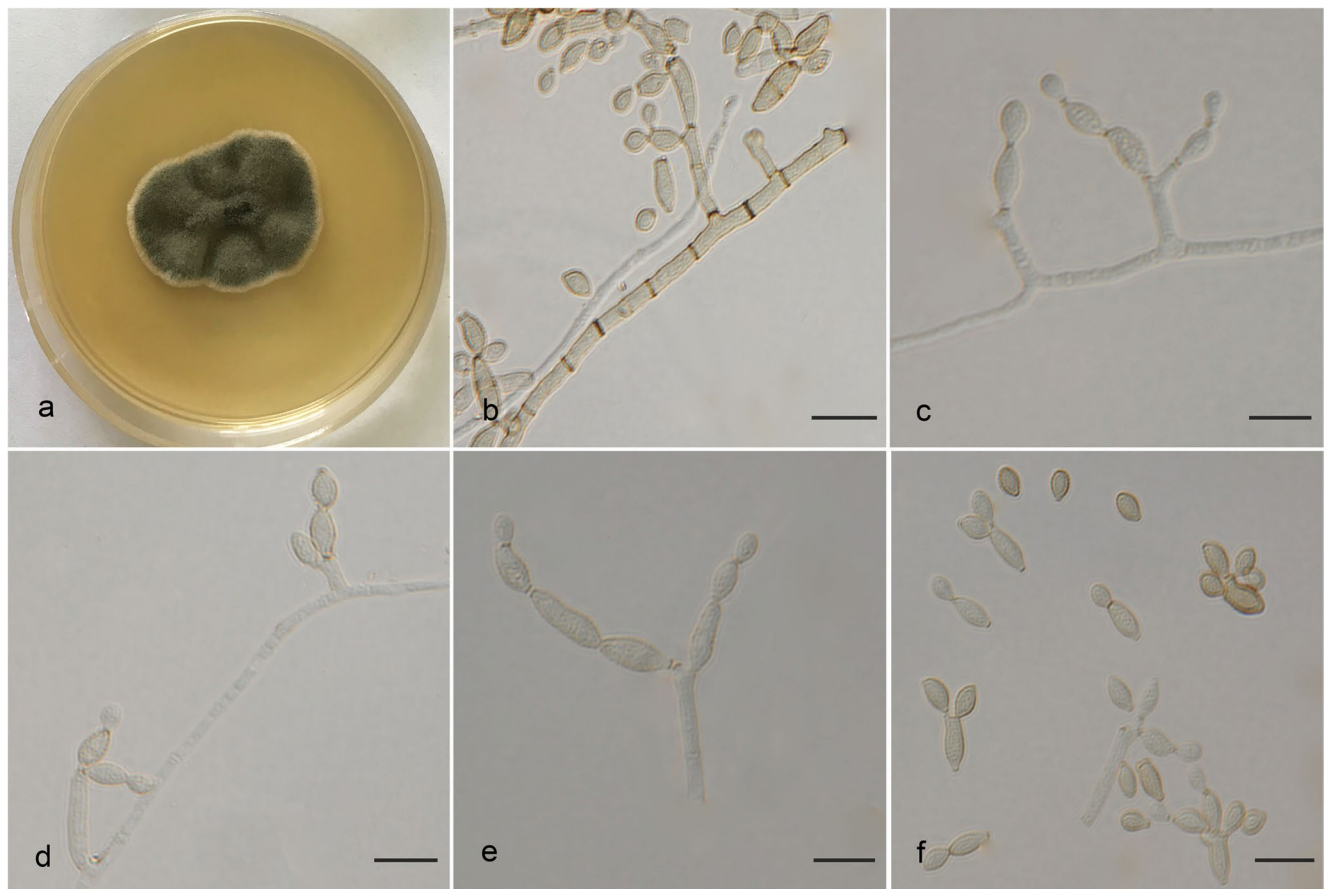


Fig. 7 *Cladosporium tenellum* (IRAN 4669 C). **a** Culture on MEA. **b–f** Conidiophores, ramoconidia and conidia. Scale bars=10 µm

in the field. Because mixed infections can produce additive or synergistic effects on symptoms and epidemic development, they may also exacerbate disease severity and alter host responses (Lamichhane and Venturi 2015). Therefore, future pathogenicity assays should also include co-inoculation designs to explore the combined effects of multiple *Cladosporium* species.

The findings in the present study will be beneficial for disease monitoring, quarantine activities, and disease management strategies. As multiple *Cladosporium* species cause leaf spot on alfalfa in Iran and some of these species have broad host ranges, this indicates a need to adjust disease management strategies. Given the micro-climatic prerequisites for infection, cultural practices that

reduce canopy humidity and leaf wetness duration (e.g., irrigation timing and canopy management) may help limit the severity of alfalfa leaf spot caused by *Cladosporium* spp., as inferred from other *Cladosporium*–foliar pathosystems (Gottwald 1985). In West Azerbaijan, symptoms were most frequently observed during the wetter, cooler part of the growing season (November–December), consistent with prolonged leaf-wetness events that favor infection. Moreover, evaluating alfalfa germplasm for resistance to *Cladosporium* species should be integrated into plant breeding programs, ensuring that resistance is effective against all identified species. In addition, because *Cladosporium* species produce diverse secondary metabolites (including mycotoxins) (Salvatore et al. 2021; Li



Fig. 8 Representative symptoms of pathogenicity test results of *Cladosporium* species on *Medicago sativa*. **a** Control plant. **b, cC.** *asperulatum* (IRAN 4656 C). **d, eC.** *limoniforme* (IRAN 4658 C). **f, gC.** *macrocarpum* (IRAN 4657 C). **h, iC.** *submersum* (IRAN 4659 C)

et al. 2024), monitoring these compounds where pathogenic species are prevalent could inform forage-quality safeguards. Finally, because *Cladosporium* assemblages and symptom expression vary across agroecosystems, targeted field validation of pathogenicity (beyond greenhouse tests) is essential to align quarantine and management recommendations with on-farm risk.

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Data availability The authors confirm that the data supporting the findings of this study are available within the article and its supplementary material. Raw data that support the findings of this study are available from the corresponding author, upon reasonable request.

Declarations

Ethical approval Not Applicable.

Conflict of interest The authors declare no conflict of interest.

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