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مقاله پژوهشی

بیماریزایی *Cladosporium halotolerans* بر روی تعدادی گیاه لگومینوز

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چکیده

در سال ۱۳۹۷ یک اپیدمی شدید بیماری برق‌زدگی نخود در شمالغرب ایران پدیدار شد. پیمایشی به منظور تعیین نژادهای عامل بیماری *Ascochyta rabiei* و تخمین خسارت به محصول انجام گردید. نمونه‌های آلوده‌ی گیاهی از یک مزرعه آلوده‌ی واقع در شهرستان پسوه برداشته شده و به آزمایشگاه مؤسسه تحقیقات کشاورزی دیم کشور، مراغه، منتقل شدند. علاوه بر *A. rabiei*، یک قارچ شبه-کلادسپوریوم از گیاهان آلوده جداسازی شدند. براساس مشخصات ریخت‌شناسی و مقایسه توالی‌های DNA، قارچ مذکور *Cladosporum halotolerans* تشخیص داده شد. آزمون‌های بیماری‌شناسی و فرضیه کخ در شرایط کنترل‌شده روی گیاه نخود انجام شد و بیماریزا بودن آن ثابت گردید. علایم بیماری پس از ۵ روز بر روی میزبان در شرایط کنترل‌شده ظاهر می‌شد که شامل زرد تا قهوه‌ای شدن برگچه‌ها بر روی انشعابات ساقه‌ی اصلی بود که به تدریج پژمرده شده و با کپک خاکستری تا سیاهرنگی پوشانده می‌شد. مطالعات هیستوپاتولوژیکی بیماریزا بودن قارچ بر روی میزبان نخود را تایید کرد. مطالعه تعیین دامنه‌ی میزبانی با استفاده از ٦ گونه گیاهی دیگر متعلق به خانواده پروانه‌سانان شامل نخود فرنگی، عدس، ماشک معمولی، ماشک ناربون، گاودانه و خلر انجام گرفت. نتایج نشان داد که قارچ عامل، قادر به ایجاد بیماری با همان علایم بر روی این گیاهان است. این پژوهش اولین تلاش برای نشان دادن وبژگی بیماریزایی قارچ *C. halotolerans* بر روی تعدادی از گیاهان لگوم در جهان است.

کلیدواژه: فاباسه، نخود، بیماریزایی، دامنه میزبانی، پسوه

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Research Article

Pathogenicity of *Cladosporium halotolerans* on some legumes

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Abstract

In 2018, a drastic Ascochyta blight epidemic on chickpea crops occurred in the Northwest of Iran. A survey was conducted in the region to identify races of *Ascochyta rabiei* and estimate the yield loss. Infected plant samples were taken from an infected field in Pasvah region and transferred to the lab at Dryland Agricultural Research Institute (DARI), Maragheh. In addition to *A. rabiei*, a Cladosporium-like taxa is also isolated from plants showing disease symptoms. Based on morphological characteristics and DNA sequence comparisons, isolates of this fungus were identified as *Cladosporium halotolerans*. Pathogenicity tests and Koch's postulates were verified and fulfilled on chickpeas under controlled conditions. In this situation, the disease symptoms were observed on the host plant by 5 days after inoculation (*dai*) as yellow-brownish leaflets on the branches. Subsequently, the infected leaves turned to wilt and were covered with gray-black mold. Histological studies also confirmed the pathogenicity of this fungus on chickpeas. Host range investigations were carried out on the further six species belonging to the Fabaceae, including garden pea, lentil, garden vetch, narbon vetch, bitter vetch, and grass pea. Results showed that this pathogen can produce the same disease symptoms on all inoculated plants as well. This study represents the first attempt to pathogenicity of *C. halotolerans* on legumes in the world.

Keywords: Fabaceae, Chickpea, Pathogenicity, Host range, Pasveh

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Introduction

The agricultural system in the drylands of Iran is based on the growth of cereals, including wheat and barley, followed by legumes and oilseeds. Chickpea (*Cicer arietinum* L.) is rankings on the top of pulse crops grown in these areas with 456,400 ha (FAO 2019) and is considered as a valuable crop stand for rotation with cereals. In the cereal-based agricultural systems of these areas, the selection range for a candidate legume crop to be included in the rotation is very limited; therefore, chickpea along with lentil, grass pea, and vetches are worthy options for the farmers (Ghaffari 2010).

Abiotic and biotic stresses are the major constraints of rainfed farming in the country (Ghaffari 2010). At a global scale, plant pathogens handle up to 16% of crop loss (Oerke 2006). Nene *et al*. (1996) have listed a total number of 49 and 172 pathogenic agents on chickpea in the years 1974 and 1995; respectively, which shows a 3.5-fold increase over 20 years. Furthermore, 27 most economically important diseases on the crop have been listed (Nene *et al*. 2012).

In the year 2018, a severe epidemic of Chickpea Ascochyta Blight (CAB) occurred in the west and northwest of the country and the disease incidence reached 100% in some regions. To identify the extent of the outbreak and to detection of involved CAB pathotypes, a survey was conducted by DARI. Besides the causal agent of CAB, a Cladosporium-like taxa was also isolated in the cultures. Therefore, the objectives of this study were to identify this fungus and its range of hosts on some legumes.

Materials and methods

Sampling, isolation, and morphological characterization

In June 2018, three symptomatic plant samples of CAB were collected from an infected field in the Pasveh region (47° 26’ 22” N, 36° 40’ 50’’ E, 1549 m), Northwest of Iran, and transferred to the laboratory. The samples were observed under a zoom stereo-microscope (Olympus, model SZH). Leaves, stems, and pods were cut into the small segments, surface-disinfected with sodium hypochlorite (0.5%) for 5-10 min, rinsed in sterile distilled water, cultured on PDA, and incubated at 25° C for 7-10 days. Colonies of *A. rabiei* along with colonies of an unknown fungus appeared after 7 days on the cultures.

Out of two isolates of the Cladosporium-like fungus, one isolate was selected for the further studies. Purification and sub-culturing of the fungus were conducted on PDA amended with a 1 mg/ml chloramphenicol. The preliminary studies using the taxonomic key of Barnett & Hunter (1972) showed that the colony belonged to *Cladosporium* sp. The pure culture was deposited in the Culture Collection of DARI (CCD-C74) and the Iranian Fungal Culture Collection (IRAN 4124C).

Morphological characterization was done on SNA incubated in the darkness at 25°C after7-9 days (Bensch *et al.* 2010, 2012, Schubert & Braun 2007). Colony color was classified on MA or PDA incubated at 25°C for 14 days (McKnight and Rayner 1972). For the macroscopic studies, small segments of the 7-days-old colony were taken, stained with lactophenol cotton blue for 5 min, mounted in 50% aq. glycerol on the microscope slides and observed under a microscope (Olympus, model BH2). Pictures were taken using the camera Nikon 1300 D. Minimum 30 replications were conducted for each fungal measurement.

Molecular characterization

Pure culture mycelium on MEA was removed from the surface of plates using a sterilized laser blade, squished in distilled sterile water, and stored at 4°C. The genomic DNA was extracted from the mycelial mat using the Chelex method (Walsh *et al.* 1991, Hirata 1996). PCR amplification conditions were as follows: an initial denaturation temperature of 94°C for 5 min, followed by 40 cycles of denaturation temperature of 94°C for 45 s, primer annealing at 48°C (52°C for *TEF*1-α and *act*) for 30 s, primer extension at 72°C for 90 s, and a final extension step at 72°C for 6 min (Bensch *et al.* 2012). The internal transcribed spacer (*ITS*), transcription elongation factor (*TEF*1-α), and Actin regions (*act*) of rDNA was amplified using the primers of *ITS*1*/ ITS*4 (White *et al.* 1990); *TEF*1-α,728F - *TEF*1-α,986R, and *act*,512F –*act*,783R (Carbone & Kohn 1999), respectively. The resulting fragments were sequenced using the Sanger method by Microsynth AG (Balgach, Switzerland). Consensus sequences for each locus

were assembled in BioEdit (Hall 1999). The generated sequences were compared with reliable sequences found in National Center for Biotechnological Information (NCBI) database (https://www.ncbi.nlm.nih.gov) using the Basic Local Alignment Search Tool (BLAST). All sequences from *ITS*1/*ITS*4, *TEF*1-α, and *act* were deposited on GeneBank as MW307298, MW326733, and JN906989; respectively. The sequences of different genes of our isolates showed 100% similarity with several ascensions of *Cladosporium halotolerans*. Sequences with high similarity belonging to the adjacent strains were added to the alignment. Subsequently, the aligned sequences were subjected to the phylogenetic analysis, and the tree was performed using the Neighbor-Joining method. All phylogenetic analyses were carried out in Mega 11 (Tamura *et al*. 2021).

Pathogenicity test

To confirm the pathogenicity, a pure fungal isolate (CCD-C74) was grown on MEA, incubated at 25ºC under a photoperiod of 12 h light / 12 h darkness for 7 days. Conidia were harvested from the surface of the media culture using a sterilized laser blade after flooding the surface with sterile distilled water. Subsequently, the conidial suspension was filtered through a four-layer cheesecloth, centrifuged at 7500 rpm for 14 min, and fixed at 1×10^5 spores per ml concentration (Meneses *et al*. 2018). Two to three drops of tween-20 per litter were added as the wetting agent. A desi type chickpea cultivar, Kaka, was used as the experimental host plant material. Seeds were surface sterilized using 0.5% sodium hypochlorite for 10-15 min, rinsed in sterile distilled water then sown in the pots of 5×8×6 cm containing field soil (sandy clay loam) and grown in a growth chamber at 20 ± 3ºC under 12 h dark/12 h light photoperiod for 3 weeks to reach to 4-6 leaf-stage. Inoculation was conducted using hand spraying, let to the drops fallen from the leaf surface, then pots were put under a plastic cover to maintain constant high humidity (> 80%) for 24 h at 25 ± 2ºC under 12 h dark/12 h light photoperiod at 250 µmol/m^2s. Six independent replications were performed and two other pots were allocated as controls, sprayed only with distilled water (sum 8 pots). The inoculated plants were visited for appropriate symptoms from the 5th *dai* onward.

Histopathological studies

Inoculated leaflets were detached at 1, 2, and 7 *dai* and fixed in the solution of glacial acetic acid: ethanol (1:1) for 24-48 h, then cleared in chloral hydrate solution (20% w/v) for 12-24 h. Subsequently, leaflets were stained with lactophenol cotton blue for 5-10 min and finally rinsed in ethanol 70% (Isenegger *et al*. 2011). Conidial germination, penetration procedure, and development of symptoms were studied in this section.

Host range assessment

Six legume species including *Pisum sativum* (garden pea), *Lens culinaris* (lentil), *Vicia sativa* (garden vetch), *V. narbonensis* (narbon vetch), *V. ervilia* (bitter vetch), and *Lathyrus sativus* (grass pea) were chosen for this purpose. Inoculation procedures were conducted on three replications each according to the method mentioned above using the same isolate.

Results

Fungal isolation and morphological identification

Principally, the plant samples had been collected based on CAB symptoms, so the probable symptoms produced by this fungus in the field conditions were might be mixed or covered by the symptoms produced by *A. rabiei*. The precise observations of the plant samples under the stereo-microscope showed that the leaf surface has been covered with a mass of gray to black mold, and white mycelia (Fig. 1 A). The fungus produced olivaceous to olivaceous-gray colonies on the surface of the media culture, olivaceous-black on the reverse, white margins, furrowed center, with profuse sporulation in the absence of aerial mycelia (Fig. 1 B, C). On SNA plates, mycelia were slightly superficial. Microscopic studies showed that the mycelium was (1–)2–3(−3.5) µm in wide, smooth, or slightly verrucose, and the walls were un-thickened. Conidiophores were solitary, often terminally, erect, straight, non-nodulous, rarely attenuated towards the apex, usually unbranched, (21−)40–130 × (2–)2.5−3 µm, pale brown, smooth to minutely verrucose, and walls were un-thickened with up to 3−4 non-

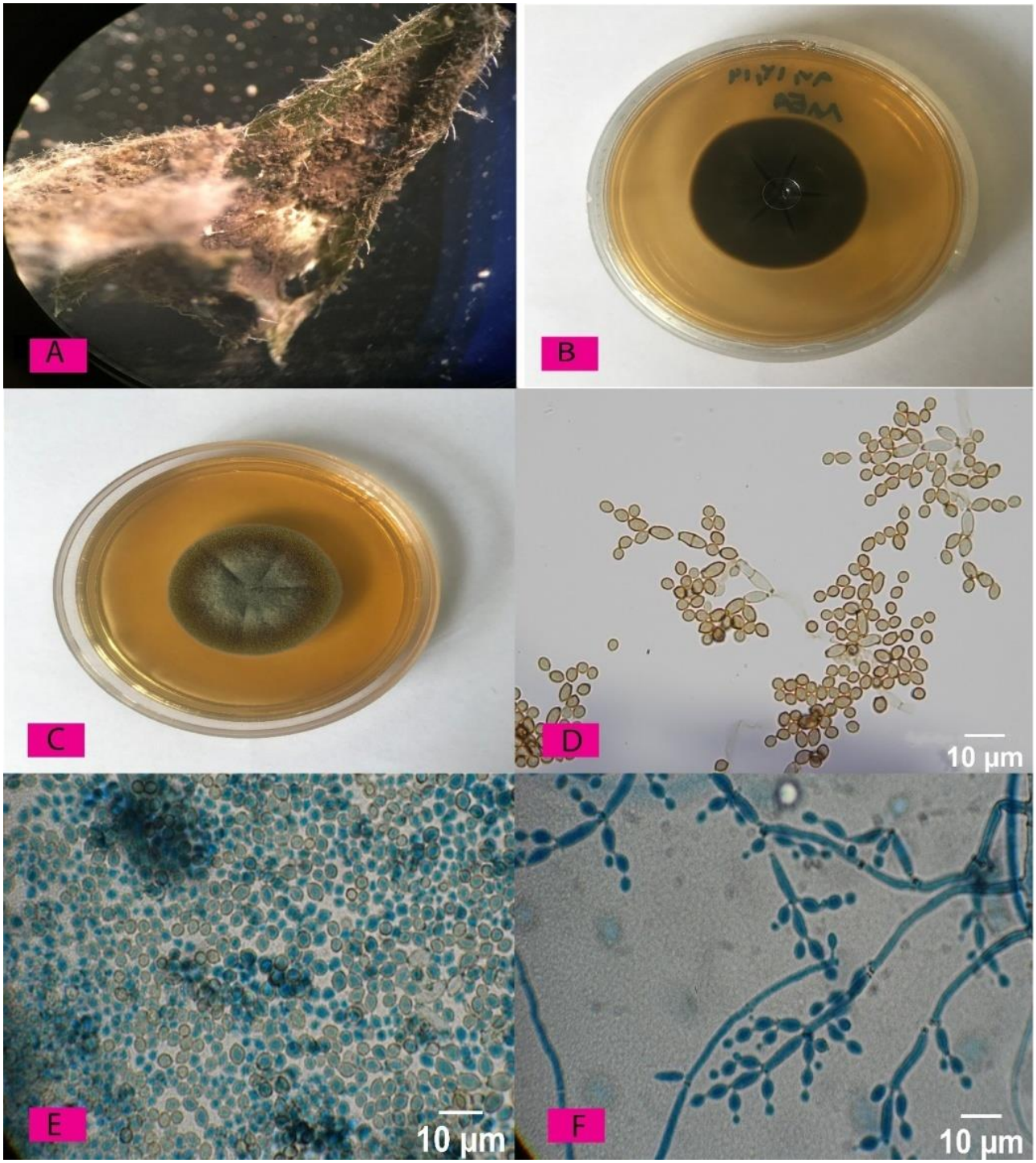

Fig 1. Symptoms of the diseases on a naturally infected chickpea plant and mycological aspects of the fungal pathogen. A: Chickpea tip leaf covered by a gray-black mold mass of *Cladosporium halotolerans*; B, C: Reverse and the surface of the colonies on MEA, respectively; D-F: Laterally conidiophores, conidiogenous cells, and conidia.

constricted septa. Conidiogenous cells were integrated, terminally or sometimes intercalary, cylindrical, 8–21(−30) µm in length, usually neither geniculate nor nodulous, up to three sub-denticulate or denticulate conidiogenous loci, 1–1.5 µm in diameter. Conidia were catenated in short branched chains, up to 4–5 conidia in the unbranched terminal part of the chain. Terminal

Table 1. The number distribution of significant aliments in the standard 100 top search produced in the BLAST process of the sequences obtained by the primers used in this study

	Primers		
Species	ITS1/ITS4	TEF1-α	act
Cladosporium halotolerans	69	95	82
Cladosporium sp.	24	0	1
Cladosporium cladosporioides	2	0	1
Cladosporium parahalotolerans	3	5	12
Cladosporium sphaerosporium	1	0	4
Unknown strain	1	0	0
Total	100	100	100

conidia were small, globose or subglobose, 2.5(–3) × 2–3 µm, and aseptate. Intercalary conidia were sub-globose, ovoid, 3–9 × (2–)2.5–3 µm, pale to medium brown. Secondary ramoconidia were ellipsoid, fusiform, 7–22(–25) × 2–2.5(–3) µm, 0–1(–3) septate, not constricted at septa. Septa were often somewhat darkened, pale to medium brown, almost smooth or minutely verrucose. Walls were un-thickened, with 3−4 distal hila, hila protuberant or denticulate, (0.5−)1–1.5 µm in diameter, thickened and darkened (Fig. 1 D, F). Based on morphological and cultural features, the fungus was identified as *Cladosporium halotolerans* Zalar, de Hoog & Gunde-Cimerman (Zalar *et al*. 2007).

Molecular identification of the fungus

Using BLASTn search tool, the sequences obtained isolates through primers *TEF*1- α, *ITS*1/*ITS*4 and *act* showed high degrees of sequence similarity (*viz*. 100, 100 and 99.35 percent) with the sequences of accessions belonged to *C. halotolerans* including MF47344, MT626047 and MF473938, respectively. The standard 100 top search results aligned from GenBank presented a different combination of sequences (Table 1).

Phylogenetic analysis

Studies with 23 type-derived sequences belonged to the nearly relative species of the genus *Cladosporium* derived from GenBank (Table 2) based on all three gene regions along with our isolate, produced a phylogenetic tree shown in Fig. 2. The isolate was placed in a unique cluster with roughly strong support 81% with other isolates of *C. halotolerans*, including MF 473441, MF 4733448, and MF473419. Altogether, the phylogenetic study revealed that the Iranian isolate is *C. halotolerans*; so, morphological and molecular identifications were confirmed and supported by this method.

Pathogenicity test and host range study

The disease symptoms were observed on chickpea plants from 5 *dai* onward (incubation period), the leaflets turned to brown-yellow, dried, and fallen which resembled the symptoms were observed in the field in composition with chickpea Ascochyta blight. The whole plant became blighted, wilted, and died (Fig. 3) under continuous humidity conditions; while the control plants remained healthy.

Histopathological observations indicated that within the first *dai*, spores have germinated on the surface of the leaflet. In 3*dai*, massive germinated spores could be visible on the leaf surface. The cuticle was completely penetrated at 7 *dai*, leaf tissue was initiated to be destroyed and colonized by the fungus. Precise observations showed that at that time, the fungus was capable to produce hypha and sporulation as the second cycle (Fig. 4). Consequently, the fungus was re-isolated at 7 *dai* from inoculated plant samples, transferred onto the MEA, and identity was confirmed based on morphological characteristics; so, Koch's postulates were performed. Host range studies indicated that the same symptoms could appear on the tested plants following inoculation and incubation at the same conditions. Different laten period was detected for garden pea, lentil, garden vetch, narbon vetch, bitter vetch, and grass pea as 7, 10, 10, 14, 9, and 9 days, respectively.

Discussion

C. halotolerans recently separated from *C. sphaerospormum* species complex (Zalar *et al.* 2007) based on morphological and molecular identities. Morphological observations, solely, are not sufficient to identify a fungus at the species level (Geiser 2004).Therefore, we used *ITS*, *TEF* 1-α and *act* primers to do molecular fingerprinting of the isolate. The efficiency of these markers have been reported previously to reveal taxonomic relationships among different species of either *Cladosporium* (Bensch *et al*. 2010, 2015) or other fungal species (Archer & Xu 202, Brazee *et al.* 2011, Divakara *et al.* 2014, Ettenauer *et al.* 2014, Huang *et al.* 2018, Karlsson *et al.* 2016, Nilsson *et*

Table 2. Host, origin, and GenBank accession numbers of *Cladosporium* spp. used in phylogenetic studies. *Cercospora beticola* was used as outgroup

No.	Species	Isolate	Host/Source	Locality	GenBank Accession No.			Reference
					*ITS*1/*ITS*4	*TEF*1-a	*act*	
1	*Cladosporium halotolerans*	DTO:160-I2	Living room	The Netherlands	MF473014	MF473441	MF473863	(Bensch *et al.* 2018)
2	*Cladosporium halotolerans*	DTO:305-E9	House dust	Mexico	MF473021	MF473448	MF473870	(Bensch *et al.* 2018)
3	*Cladosporium halotolerans*	CPC:22308	Indoor air sample	USA	MF472991	MF473419	MF473840	(Bensch *et al.* 2018)
4	*Cladosporium halotolerans*	CCD-C74	*Cicer arietinum*	Iran	MW307298	MW326733	JN906989	Current study
5	*Cladosporium globisporum*	CPC:19124	Indoor environment	Denmark	MF472985	MF473413	MF473834	(Bensch *et al.* 2010)
	Cladosporium globisporum	CBS:812.96	Meat stamp	Sweden	HM148096	HM1483340	HM148585	(Bensch *et al.* 2010)
6	*Cladosporium acalyphae*	CBS 125982	*Acalypha australis*	South Korea	HM147994	MF148235	HM148481	(Bensch *et al.* 2010)
7	*Cladosporium aciculare*	CPC:16547	*Syzygium coryanthum*	Australia	KT600411	KT600509	KT600607	(Bensch *et al.* 2015)
8	*Cladosporium aerium*	DTO:323-G7	Indoor air sample	China	MF472899	MF473326	MF473749	(Bensch *et al.* 2018)
9	*Cladosporium aggregatocicatricatum*	CBS:284.84	Tempeh	The Netherlands	KT600450	KT600549	KT600647	(Bensch *et al.* 2015)
10	*Cladosporium allicinum*	CPC:12212	*Hordeum vulgare*	Belgium	EF679351	EF679426	EF67950	(Schubert *et al.* 2007)
11	*Cladosporium angulosum*	CPC:22271	Indoor air sample	USA	MF472918	MF473345	MF473768	(Bensch *et al.* 2018)
12	*Cladosporium colocasiae*	CBS:386.64	*Colocasia esculenta*	Fiji	AY251075	HM148308	HM148553	(Bensch *et al.* 2010)
13	*Cladosporium dominicanum*	CPS:20109	Unknown vine	Taiwan	KT600391	KT600488	KT600586	(Bensch *et al.* 2015)
14	*Cladosporium gamsianum*	CBS:125989	*Strelizia* sp.	South Africa	M148095	HM148339	HM148584	(Bensch *et al.* 2010)
15	*Cladosporium pseudocladosporioides*	CPC:14382	*Acer macrophyllum*	Canada	HM148190	MH148434	HM148679	(Bensch *et al.* 2010)
16	*Cladosporium perangustum*	CPC:13686	*Eucalyptus placita*	Australia	HM148138	HM148382	HM148627	(Bensch *et al.* 2010)
17	*Cladosporium ramotenellum*	CBS:121628	Hypersaline water	Slovonia	EF679384	EF679462	EF679538	(Schubert *et al.* 2007)
18	*Cladosporium sphaerospermum*	CBS 193.54	Man, nails	The Netherlands	DQ780343	EU570261	EF101380	(Dugan *et al.* 2008)
19	*Cladosporium tenuissimum*	CBS 125995	*Lagerstroemia* sp.	USA	HM148197	HM148442	HM148687	(Bensch *et al.* 2010)
20	*Cladosporium xylophilum*	CPC:16356	*Musa* sp.	Mexico	KT600460	KT600559	KT600657	(Bensch *et al.* 2015)
21	*Cladosporium subinflatum*	CPC:15565	*Iris* sp.	Ukraine	KT600447	KT600546	KT600644	(Bensch *et al.* 2015)
22	*Cladosporium langeronii*	CBS:189.54	Man, ulcero-nodular mycosis of arm and hand	Brazil	DQ780379	JN906990	EF101357	(Bensch *et al.* 2012)
23	*Cercospora beticoal*	CBS117:47	*Beta vulgaris*	Czechia	DQ233322	Q233348	DQ233374	(Groenewald *et al.* 2006)

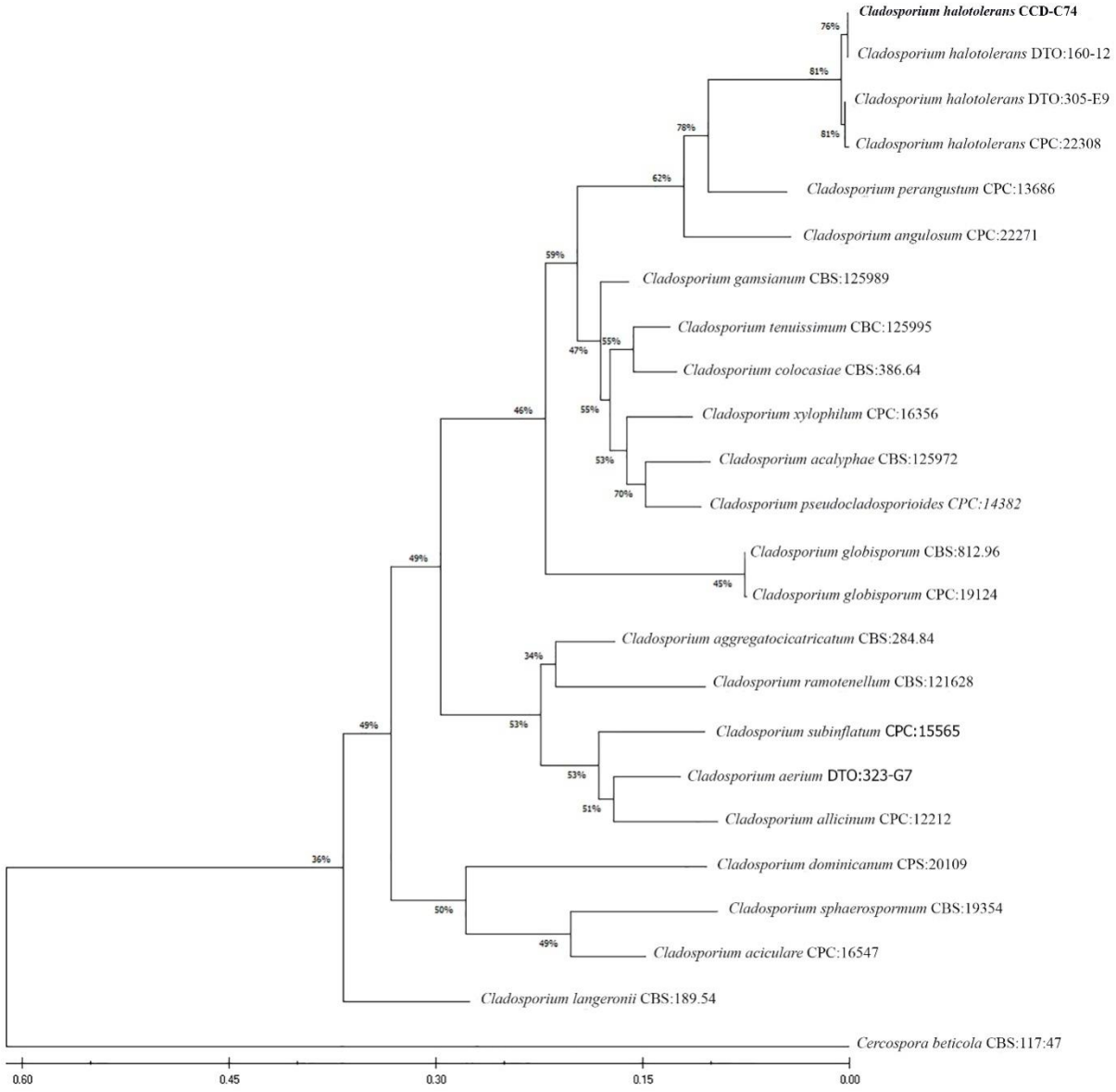

Fig 2. Phylogenetic tree of combined *TEF*1-α, *act*, and *ITS* sequences for *Cladosporium* species derived from GenBank along with the studied isolate of *Cladosporium halotolerans* (in bold) based on a Neighbour Joining analysis with 1000 bootstrap replications. The tree was rooted with *Cercospora beticola*

al. 2008, Schoch *et al.* 2012). Based on morphological and molecular methods, our isolate obtained from chickpea was identifies as *C. halotolerans* (Zalar *et al*. 2007).

Cladosporium is known as a cosmopolitan fungal genus, lives in indoor environments; on plants, fungi, and animals. Frequently, it has been isolated from soil, food, paint, textiles, and other organic matters (Bensch *et al.* 2012). For the first time, *C. halotolerans* isolated from Great Salt Lake, USA (Baxter & Zalar 2019). Subsequently, it has been reported from various environments such as indoor and clinical habitats (Sandoval-Denis *et al.* 2015, Segers *et al.* 2016, Yang *et al.*, 2016), desert rocks (Gonçalves *et al.* 2016), and arctic ice (Gunde-Cimerman *et al.* 2003). Furthermore, different biological properties were recorded for it, *e.g.* being as endophyte in *Vitis*

Fig 3. Disease symptoms caused by *Cladosporium halotolerans* on chickpea under controlled conditions, A: Yellow-brownish leaflets on the branch appeared at 5 *dai*; B: Wilted leaflets covered with gray-black mold at 10 *dai* under continuous humid conditions

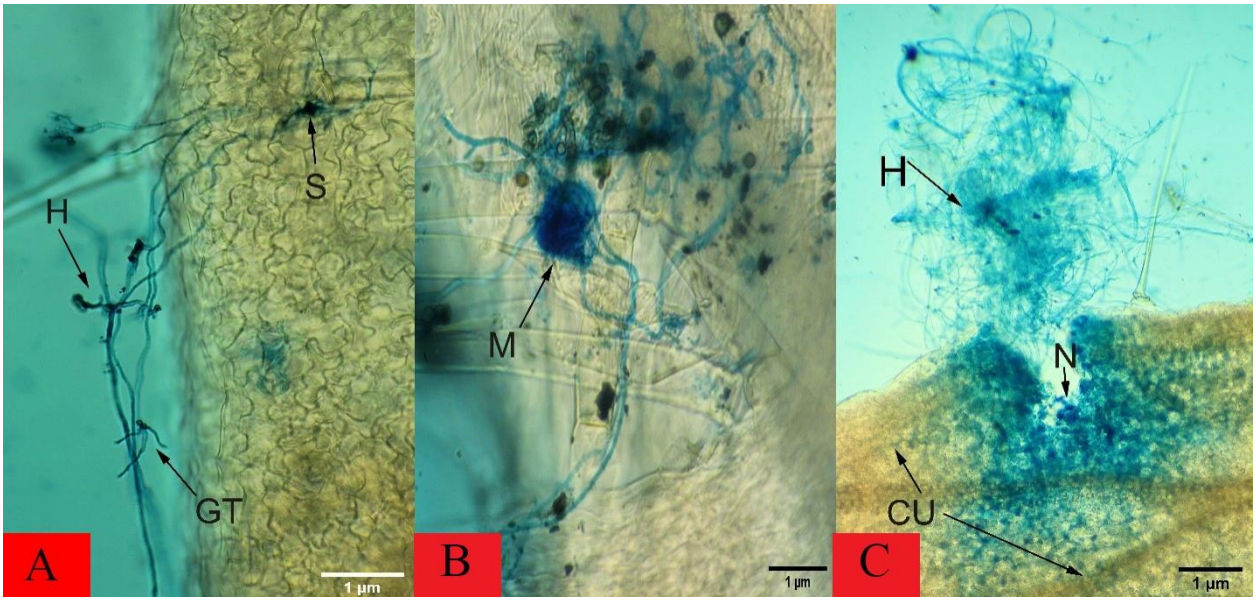

Fig 4. Histopathological events of *Cladosporium halotolerans* on the leaf segments of chickpea plant; A: Germinated spores (S) produced germ tube (GT) and hyphae (H) on the surface of the inoculated leaf at 1 *dai*, there was no specific attention into the stomata, B: Development of hyphae on the leaf surface and producing mycelial mass (M) at 3 *dai*; C: The pathogen penetrated the cuticle (CU) and caused a necrotic hole (N) where a skein of hyphae (H) was produced at 7 *dai*

labrusca (de Oliveria *et al.* 2014), animal (dog) pathogen (Headley *et al.* 2019), and post-harvest rotten agents on the dried date (Quaglia *et al.* 2020) or grapevine (Lorenzini & Zapparoli 2015).

Although *Cladosporium* spp. has already been reported in association with seeds of chickpea (Kandhare 2014) and lentil (Kaiser 1992); there is not any report of the fungus as a plant pathogen so far. In Iran, it has been reported as a saprobe or secondary invader on decaying stems or leaves of *Citrus* sp., *Pedilanthus* sp., *Acer velutinum*, *Phragmites* sp., *Rubus* sp., *Phoenix dactylifera*, *Fragaria ananassa*, *Phaseolus vulgaris* and *Nerium oleander* (Amirmijani *et al*. 2014).

The evolutionary history of many fungal pathogens is begin with a jump in the host range which can take place for the native hosts and fungal species as well as for the introduced pathogens (Burdon *et al*. 2009). Tack *et al*. (2012) using 48 studies on 30 distinct host-pathogen systems demonstrated that the changes in the pathogenicity is universal across multiple spatial and temporal scales, and could have either ecological or genetical reasons. In this study, we showed that an isolate of *C. halotolerans* which co-exist with *A. rabiei* on chickpea host, although known as a saprophytic fungus, has been appeared as a plant pathogen; not only on the chickpea, but also on the further seven species of Fabaceae. Shifting of a fungal species from saprophytic form into a pathogenic form, has already been reported for *Alternaria* spp. (Thomma 2003). The phenomenon which observed in this study must have a scientific reason that need to be studied later.

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