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Research Article

Occurrence of jujube brown spot disease in Iran

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Abstract
Jujube tree has a natural distribution in tropical and sub-tropical regions of Asia. Symptoms of brown-to-black spots on leaves, and fruits, and twigs blight were observed in the hills of the suburbs of Nurabad County, Fars Province, Iran, in 2022. This research was conducted to identify the cause of this disease based on morphological and genetic characteristics. The diseased leaves and branches of the neighboring trees in this area were sampled. The pathogen was isolated and purified after surface disinfection of disease tissues on potato/dextrose/agar medium. Its morphological characteristics were studied and the fungus *Nothophoma quercina* was identified. Phylogenetic analysis base on the comparison of beta-tubulin (tub2), and ITS-rDNA genes sequences, with related fungi in NCBI Gen Bank, confirmed the of *N. quercina* species. Its pathogenicity was proved on the side cut jujube branches based on Koch's postulates in vitro. This is the first report of brown spot and twigs blight of the jujube trees caused by *N. quercina* in Iran.
Keywords: β-tubulin, ITS-rDNA, *Nothophoma*, *Zizyphus*

مقاله پژوهشی

وقوع بیماری لکه قهوه‌ای کُنار در ایران

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چکیده
درخت کُنار در مناطق گرمسیری و نیمه‌گرمسیری آسیا گسترش طبیعی دارد. نشانه‌های لکه‌های قهوه‌ای تا سیاه‌رنگ روی برگ‌ها و میوه‌ها و سوختگی سرشاخه‌های کنار، در تپه‌های حومه شهرستان نورآباد استان فارس ایران در سال ۱۴۰۱ مشاهده شد. این پژوهش برای شناسایی عامل این بیماری براساس خصوصیات ریختی و ژنتیکی انجام شد. برگها و شاخه‌های بیمار درختان کنار در این منطقه

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نمونه‌برداری شدند. بیمارگر پس از ضدعفونی سطحی بافتهای بیمار روی محیط سیب-زمینی/دکستروز/آگار جداسازی و خالص‌سازی شد. خصوصیات ریختی آن مورد مطالعه قرار گرفت و قارچ *Nothophoma quercina* شناخته شد. تجزیه و تحلیل فیلوژنتیکی آن براساس مقایسه توالی‌های ژن‌های بتاتوبولین (*tub2*) و ITS-rDNA با قارچهای مشابه در بانک ژن NCBI، گونه *Nothophoma quercina* را تایید نمود. اثبات بیماریزایی آن روی شاخه‌های بریده کنار براساس اصول کخ در شرایط آزمایشگاهی انجام شد. این اولین گزارش از لکه قهوه‌ای و سوختگی سرشاخه درختان کُنار ناشی از *N. quercina* در ایران است.

واژگان کلیدی: بتاتوبولین، ITS-rDNA ، *Nothophoma*، *Zizyphus*

Introduction مقدمه

Jujube (*Zizyphus mauritiana* Lamk.), a member of the family *Rhamnaceae*, is natural to Southeast Asia, Asia Minor, China and Caucasia. This tree is indigenous to China with a history of over 4000 years (Li et al. 2007). In Iran, it wildly grows in the north, north-east, central and southern parts of the country. Jujube is becoming gradually more popular due to its high therapeutic value, health benefits and high adaptation to various soil pH and drought conditions (Liu 2009). Several diseases are reported on jujube such as brown spot, rust, jujube anthracnose, Ascochyta blight, witches broom, and bacterial fruit soft rot (Zhang et al. 2013, Jianyu et al. 2016, Jianyu et al. 2016).

The genus *Nothophoma* was introduced by Chen et al (2015) and caused brown spot of jujube. This genus includes a variety of plant pathogens, comprising *N. infossa* (Ellis & Everh.) Qian Chen & L. Cai, *N. quercina* (Syd. & P. Syd.) Qian Chen & L. Cai and *N. gossypiicola (*Gruyter) Qian Chen & L. Cai. (Chen et al. 2015, Crous et al. 2017, Valenzuela-Lopez et al. 2018, Chethana et al. 2019, Zhang et al. 2020). *Nothophoma quercina* has been reported as the cause of jujube brown leaf spot in China (Jianyu et al. 2016). In the course of a study conducted by Khodaei et al. (2020) on different trees in Iran, this species was isolated and reported from several hosts such as *Salix alba*, *Rosa canina*, *Morus alba* in East and West Azerbaijan.

Brown spot and twigs blight were observed on jujube in the hills of the suburbs of Nurabad County, Fars Province, Iran, in the autumn of 2022. Therefore, this research was conducted to identify the cause of this disease based on morphological and genetic characteristics.

Materials and Methods مواد و روش‌ها

Pathogen isolation and morphological identification

Infected leaves, fruits and branches of jujube were sampled from this region, and was taken away to the laboratory in polyethylene bags and kept in the refrigerator (4°C). To isolate disease agent, the samples were cut into 5–10 cm segments with a sterile scalpel, sterilized in 70% ethanol for 60 seconds, rinsed in sterile deionized water, placed in glass Petri dishes without culture media, and kept under high humidity, then examined daily until the conidia were released from Pycnidia. The conidia were spread by a sterile loop on 2% water-agar medium and incubated at 25°C. After 24 hr, single-germinated conidia were transferred to PDA media (potato dextrose agar; 12 g/l potato extract, 10

g/l dextrose, 1.2% agar, with 50 µg of modified kanamycin to prevent bacterial contamination). The pure culture of isolates was used for morphological and molecular studies. Isolates were incubated on PDA at 25°C. The colors of each colony were recorded after 7 days at 25°C. After 15 days, black pycnidia were then observed, 1 ml of sterile water was added on the face of each plate, and conidia were released from pycnidia and then collected for further measurements (Zou et al. 2021). Morphological features were evaluated with the Olympus DP72 camera on the Olympus BX51 microscope (Tokyo, Japan). Measurements were performed with the Cell Sense Entry measurement module. Based on at least 50 microscopic measurements, the dimensions of each fungal structure were calculated.

Molecular characterization of pathogen and phylogenetic analysis

Selected fungal isolates were grown on PDA and incubated at 25°C for one week. Produced mycelia were scraped from the surface of cultures and ground to a fine powder in liquid nitrogen, for DNA extraction. Genomic DNA was extracted with the CTAB protocol defined in detail by Murray and Thompson (1980). DNA samples were visualized on 1.2 % agarose gels, stained with ethidium bromide (50 µg/ml), and then kept at –20°C until used for PCR amplification. Three representative isolates Iran-Nq1 to Iran-Nq3 were selected, for molecular characterization. Molecular identification was performed using partial sequences of *tub2* gene with primers bt2a and bt2b (Glass and Donaldson 1995) and ITS-rDNA region with the primer pairs ITS1 and ITS4 (White *et al*. 1990). PCR amplifications were performed by Jianyu et al. (2016) method. Genes sequencing was performed in both directions by the DNA Sequencing Service of Macrogen Co. (South Korea). *Tub2* and ITS-rDNA sequences were blasted separately using Megablast to identify their closest neighbors. *Tub2* and ITS-rDNA sequences obtained in this study were combined with sequences of representative taxa published previously by Zou et al. (2021), to determine the taxonomic status of the *Nothophoma* isolates on jujube. New nucleotide sequences were deposited in NCBI gen bank (http://www.ncbi.nlm.nih.gov).

Nucleotide sequences were edited by BioEdit v. 7.2.5 (Hall 2012) and aligned using MAFFT v.7 (Katoh and Standley 2013). Multi-locus phylogenetic Bayesian Inference (BI) analyses were carried out using MrBayes v3.2.2 (Ronquist et al. 2012). *Tub2* and ITS-rDNA sequences were divided to two partitions, loaded the concatenated sequence into Jmodel test, and chose the best model for all partitions. Four Markov chains were run for 10,000,000 generations, with a burn-in fraction set to 0.25. Maximum parsimony (MP) phylogenies were calculated with heuristic searches in PAUP v. 4.0a133 (Swofford 2002). Bootstrap analysis of 1000 replicates was confirmed the support of the branches and shown next to the branches. *Leptosphaeria doliolum* (Pers.) Ces. & De Not.was used as outgroup.

Pathogenicity test

Isolate Iran-Nq1 tested for their ability to cause symptoms on detached jujube branches. The detached stems from healthy and actively growing jujube trees were collected from Nurabad hills located in Fars province. The branches were trimmed to remove leaves and lateral shoots, and were cut to segments 20–25 cm long and 1–1.5 cm in diameter. The branches were washed and disinfected bark surfaces by spraying with 70% ethanol.

The detached branches were sealed at both ends with warm melted paraffin wax to reduce dehydration during the incubation period. Three T-shaped cuts (20–30 mm) were made in the bark with a scalpel. A 6 mm PDA mycelial plug from actively growing hyphae of *Nothophoma* isolate was placed into the cut section, the bark replaced, and the inoculated part was wrapped with Parafilm. Control-detached branches were treated only with a PDA plug without fungal isolate. Inoculated and control branches were incubated in humid chambers (plastic containers, with 100% relative humidity obtained by adding 250–300 ml water) at room temperature in the dark. The lesion development was examined after 40 days of incubation (Ghaderi and Habibi 2021).

Results and Discussion یافته‌ها و بحث

Morphological characteristics of the pathogen

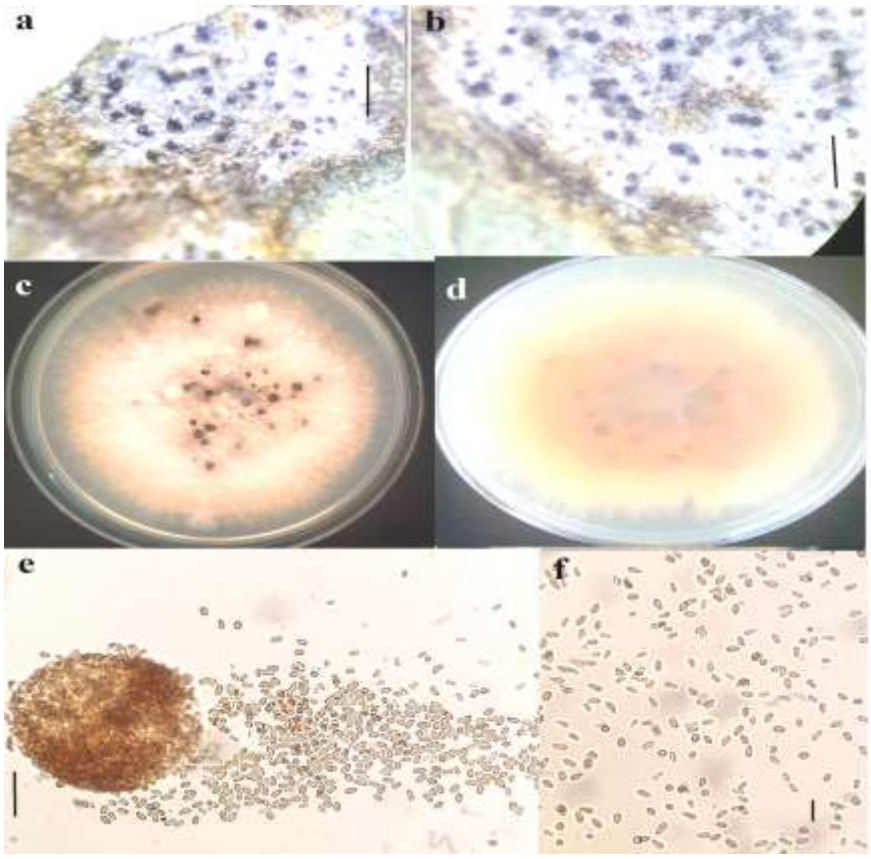
Approximately 15-20% of the jujube trees showed disease symptoms. The main symptoms were usually twigs blight, brown spots on leaves and fruits along with black pycnidia surrounded by dark-brown margins, and eventually dieback which eventually led to tree mortality. It should be noted that younger trees showed severe symptoms than older trees in Nurabad hills from Fars province, Iran. (Fig. 1).

Sample collections were carried out between early winter and late winter. An average of 50 trees were sampled from Nurabad hills with 10 tissue pieces sampled per tree. In total, 20 isolates were recovered from the infected symptomatic jujube trees; 12 isolates of leaves, 6 isolates from fruits and 2 isolates from twigs. All isolates identified as *Nothophoma quercina* based on morphological characterization.

The colonies on PDA medium were white with center greenish olivaceous to olivaceous, with pure white aerial mycelium. After 12 days, the fungus colony filled the entire Petri dishs. (Fig. 2). Pycnidia were scattered, black, solitary, globose, peroblate to suboblate, on the surface of media 60.5-140.3 µm (n=50) in diameter (Fig. 2). Conidia from pycnidia were hyaline, aseptate, subglobose to oval or obtuse in shape, a diameter of 4–7.5 × 3–4.6 µm (n=50) (Fig. 2). The maximum, minimum, and optimum temperatures for colony growth on PDA medium were 15, 27, and 40 °C, respectively.

شکل ۱. علائم لکه قهوه‌ای درخت کُنار ناشی از *Nothophoma quercina* (a) خشکیدگی شاخه‌های درخت کنار (b) برگ‌های آلوده با پیکنیدیوم سیاه‌رنگ (c) لکه‌های قهوه‌ای روی میوه.

Figure 1. The symptoms of brown spot of jujube (*Ziziphus mauritiana*) caused by *Nothophoma quercina*: (a) Dieback of jujube trees; (b) Infected leaves with black pycnidia that surrounded by dark-brown margins; (c) Brown spots on fruit.



شکل ۲. *Nothophoma quercina* مرتبط با لکه قهوه‌ای و بلایت شاخه درخت کنار: (a, b) آلودگی طبیعی برگ‌های درخت کنار، (c, d) ویژگی‌های پرگنه قارچ روی سطح بالایی و سطح وارونه روی محیط کشت PDA بعد از ۱۰ روز (e) پیکنیدیوم و کنیدیوم روی محیط کشتPDA بعد از ۱۰ روز (f) کنیدیوم‌ها.

Fig 2. *Nothophoma quercina* associated with brown spot and twig blight on jujuba: (a, b) Natural infections on leaves of *Z. mauritiana*. (c) Characteristics of colony on the upper surface of a ten-day culture on PDA media. (d) Reverse characteristics of colony on a ten-day culture on PDA media (e) Pycnidia and conidia of *N. quercina* of a ten-day culture on PDA media. (f) Conidia. Scale bars (a, b) = 100µm, (e)= 20µm, (f)=10 µm.

Phylogenetic affinity of the pathogen

PCR amplification was successful for 10 isolates and produced approximately 295 bp amplicons for *tub2* and a 510 bp amplicons for the ITS-rDNA region. Sequencing was carried out for three isolates Iran-Nq1 to Iran-Nq3. The obtained sequences of three isolates were submitted to NCBI gen bank under the following accession numbers: OQ656700 to OQ656702 for *tub2* and OQ651970 to OQ651972 for ITS-rDNA region (Table 1). The aligned data sets for *tub2* and ITS-rDNA and consisted of 337 and 515 characters. The aligned multigene data set of taxa contained 854 characters. The two phylogenetic analysis methods, BI and MP, generated trees with similar topologies. The most appropriate model chosen by jModelTest based on BIC was the general time reversible nucleotide substitution model with gamma-distributed rate variation and a proportion of invariable sites (GTR+I+G). The topology and branch lengths of the

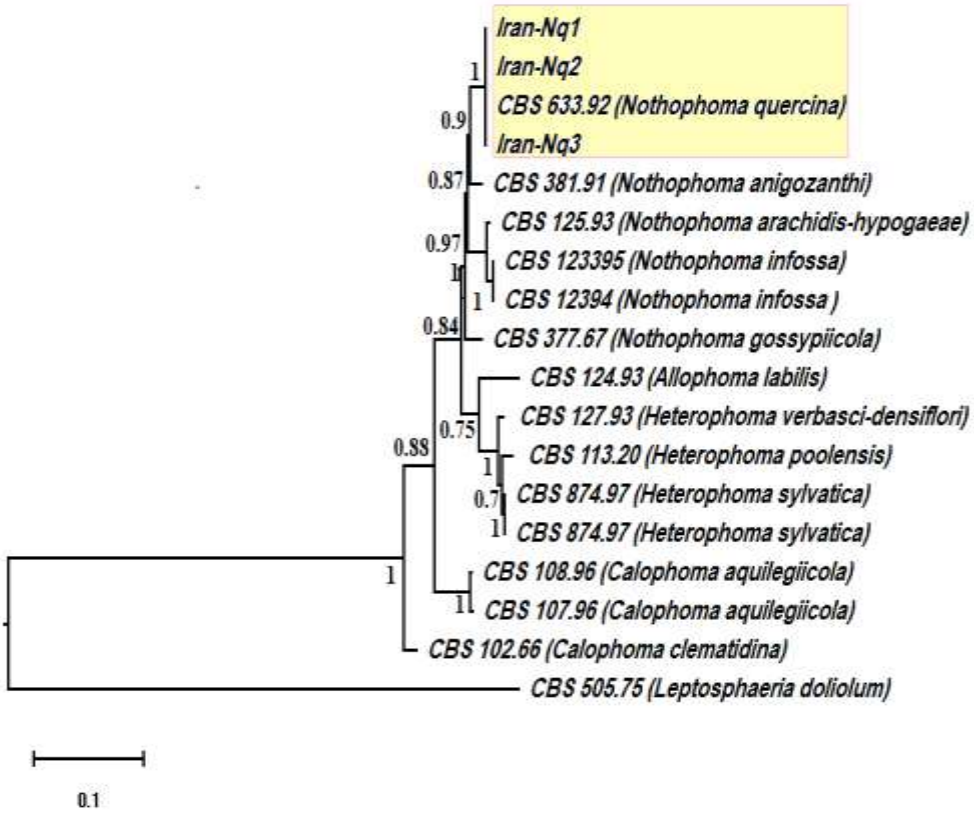
جدول ۱. جدایه‌های قارچهای از بانک ژن NCBI استفاده شده در واکاوی تبارزایی جدایـه‌هـای قـارچ جداشده از درختان کنار بیمار در این پژوهش.

Table 1. Table 1. Fungal isolates from the NCBI gene bank used in phylogenetically analysis of the fungus isolates from jujube diseased trees in this study (Iranian isolates are shown in bold type).

Species name	**strain**	**GenBank accession no.**	
		tub2	**ITS-rDNA**
Calophoma aquilegiicola	CBS 108.96	GU237582	GU237736
Calophoma aquilegiicola	CBS 107.96	GU237581	GU237735
Nothophoma gossypiicola	CBS 377.67	GU237611	GU237845
Calophoma clematidina	CBS 102.66	FJ427099	FJ426988
Nothophoma anigozanthi	CBS 381.91	GU237580	GU237852
Nothophoma quercina	CBS 633.92	GU237609	GU237900
Nothophoma arachidis-hypogaeae	CBS 125.93	GU237583	GU237771
Nothophoma infossa	CBS 123394	FJ427134	FJ427024
Nothophoma infossa	CBS 123395	FJ427135	FJ427025
Heterophoma poolensis	CBS 113.20	GU237638	GU237751
Heterophoma verbasci-densiflori	CBS 127.93	GU237639	GU237774
Heterophoma sylvatica	CBS 874.97	GU237662	GU237907
Allophoma labilis	CBS 124.93	GU237619	GU237765
Leptosphaeria doliolum	CBS 505.75	JF740144	CBS 505.75
Nothophoma quercina	**Iran-Nq1**	**OQ656700**	**OQ651970**
Nothophoma quercina	**Iran-Nq2**	**OQ656701**	**OQ651971**
Nothophoma quercina	**Iran-Nq3**	**OQ656702**	**OQ651972**

phylogenetic inferences are shown in Figure 3, that shows the phylogenetic position of isolates Iran-Nq1 to Iran-Nq3 using a Bayesian analysis of the combined data set of *tub2* and ITS-rDNA sequences. Isolates Iran-Nq1 to Iran-Nq3 grouped with *N. quercina* in a well-supported clade (posterior probability = 100).

The genus *Phoma* is a polyphyletic group with species distributed in six families in *Pleosporales* (Aveskamp et al. 2008). A phylogenetic study by Chen et al. (2015) revealed the classification within the genus *Phoma* based on multilocus phylogenetic analysis of four loci (LSU, ITS, RPB2, TUB 2) and determined that only the ITS-rDNA region sequence data is not sufficient to distinguish the *Phoma* species(Chen et al. 2015). We used *tub2* and ITS-rDNA in phylogenetic analysis and these results were consistent with the finding of Chen et al. (2015) and Jianyu et al. (2016). *Phoma fungicola* belongs to the genus *Nothophoma*, and the current name is *Nothophoma quercina*. *Phoma fungicola* has been reported to cause fruit blight and stem blight of pistachio in Arizona (Chen et al. 2013), and dieback of olive trees in Tunisia (Krid Hadj Taieb et al. 2014). So far, there has been no report of *N. quercina* species on *Z. mauritiana* in Iran (Ershad 2022). It is worth mentioning that *N. quercina* can be the causal agent of brown spot and twigs blight on *Z. mauritiana* based on morphological and molecular diagnostics. In present study, we reported the association of *N. quercina* with spot and twigs blight of *Z. mauritiana*, not previously reported in Iran. *Z. mauritiana* is one of the important trees for Fars's forests Iran. Therefore, the risk of this pathogen needs further exploration, and effective control measures should be made.

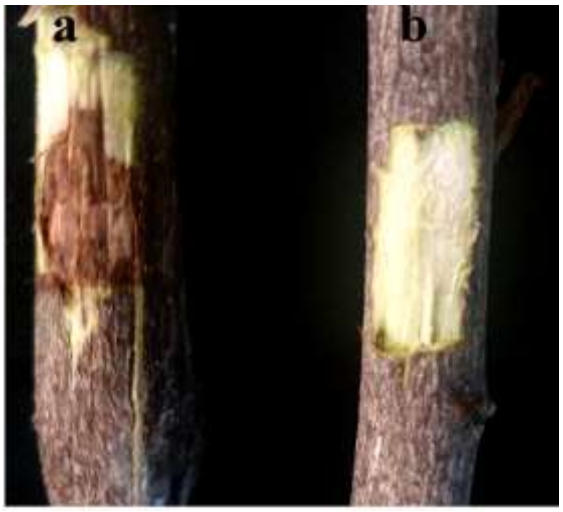


شکل ۳. درخت فیلوژنیکی (Bayesian inference) از یازده گونه *Phoma* spp. بر اساس ژن بتاتوبولین و ناحیه ITS-rDNA می‌باشد. توالی‌های استفاده شده در این مطالعه در جعبه قهوه‌ای رنگی مشخص شده است. مقدار اعتبارسنجی در شاخه‌های درخت (۰ تا ۱) نمایش داده شده است. از گونه *Leptosphaeria doliolum* به عنوان آرایه خارج از گروه استفاده شد.

Figure 3. Phylogram derived from Bayesian inference analysis of *tub2* and ITS-rDNA data set of 11 *Phoma* species, nucleotide sequences of the current study were enclosed in a brown box. Bayesian posterior probabilities (0-1) are indicated at the nodes. *Leptosphaeria doliolum* is used as outgroup taxon.

Pathogenicity test

Nothophoma quercina was pathogenic on detached branches of jujube and caused necrotic lesions after 40 days of inoculation. In general, infected branches showed necrotic lesions on the bark that extended into the wood. No lesions were observed in non-inoculated control branches (Fig. 4). Inoculated isolates were reisolated from the lesions but not from controls and isolates were identical to the original isolate based on the cultural and morphological characteristics. Koch's postulates were completed and confirmed that *N. quercina* was responsible for branch blight in Iran. To our knowledge, this is a new report of *N. quercina* associated with brown spot and twig blight on *Z. mauritiana* in Iran. Consequently, our report would be useful for its management.



شکل ۴. شاخه‌های بریده از درخت کنار، ۴۰ روز بعد از مایه‌زنی با *Nothophoma quercina* ، لکه-های نکروتیک را نشان می‌دهند: a) ایزوله Iran-Nq1 از گونه *Nothophoma quercina* ، b) شاهد

Figure 4. Detached branches of jujube trees (*Ziziphus mauritiana*) showing necrotic lesions of the inner bark after 40 days after inoculation with *Nothophoma quercina*: (a) isolate Iran-Nq1 of *Nothophoma quercina*, (b) control.

Conclusion نتیجه‌گیری

Brown spot and branch blight is present in Nurabad hills from Fars province. This is the first report of brown spot and twig blight of jujube trees caused by *Nothophoma quercina* in Iran.

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