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Research Article

MORPHOLOGICAL AND MOLECULAR IDENTIFICATION OF *ALTERNARIA DUMOSA* CAUSING POST-HARVEST BLACK SPOT DISEASE IN PERSIMMON (*DIOSPYROS KAKI* L.) IN PAKISTAN

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ABSTRACT

A survey was conducted from October to January 2021 in local fruit markets in Lahore and surrounding areas to collect persimmon fruit (*Diospyros kaki*) displaying small black spots, ranging from 3-5 mm in size. To isolate the pathogen, tissue segments (3-5 mm) from the symptomatic fruit were placed on Malt Extract Agar and incubated at 28°C. After 7 days, light brown mycelia appeared. Based on morphological characteristics, the preliminary pathogen was tentatively identified as *Alternaria dumosa*. To confirm the identification, genomic DNA was extracted and amplified using primer pairs for ITS, GAPDH, Calmodulin, Actin, and TEF. The resulting sequences were compared to those in GenBank using BLAST searches in NCBI, confirming a close match to *A. dumosa*. The sequences were deposited in GenBank under accession numbers OK447926, OL754642, OL804135, OL830275, and OL962418. For pathogenicity testing, surface-sterilized, uninfected persimmon fruits were inoculated with a conidial suspension (10^6 spores/ml) of the isolate from this study, while sterile distilled water was used for the control treatment. The fruits were then incubated at 28°C for 7 days. The experiment was repeated twice for validation. Pathogenicity tests showed that 85% of the inoculated persimmon fruits developed characteristic black spots within 7 days, indicating the high virulence of *A. dumosa* under controlled conditions. The inoculated fruits displayed symptoms similar to those observed in naturally infected fruits, while the control fruits remained symptom-free. Based on these results, we concluded that *A. dumosa* is the causative agent of leaf spot disease in persimmon fruit.

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INTRODUCTION

Persimmon fruit (*Diospyros kaki*) is a nutritionally rich species widely cultivated in Asia, South America, and the Mediterranean regions (Senica et al., 2016). In Pakistan, it thrives in regions with favorable agro-climatic conditions, such as the hilly areas of Khyber

Pakhtunkhwa, Azad Jammu and Kashmir, and parts of Punjab. Persimmon fruits are highly valued for their rich content of essential nutrients, bioactive compounds, and health-promoting properties. These include vitamins, minerals, dietary fiber, terpenoids, polyphenols, flavonoids, steroids, and carotenoids, all of which offer considerable potential for enhancing human health (Pérez-Burillo et al., 2018). Over the past two decades, global persimmon production has increased fivefold, driven by the expansion of cultivation areas (FAO, 2019). Despite its growing popularity, persimmon cultivation faces significant challenges due to its susceptibility to various microorganisms, particularly fungal pathogens, which lead to substantial economic losses (Savary et al., 2019). Persimmon is vulnerable to a range of diseases, including fruit, foliar, and wood pathogens, as well as postharvest diseases (Alves et al., 2021; Evallo et al., 2022). Among these, *Alternaria* black spot disease (ABS), caused by *Alternaria alternata*, is a major contributor to fruit losses (Palou et al., 2012; Dash et al., 2022). This pathogen is particularly destructive due to its ability to sporulate and resist many postharvest management practices. It can infect fruits at any stage, from cultivation to marketing (Chaerani et al., 2007; Kokaeva et al., 2015).

Economic losses from postharvest diseases are especially severe when latent fungal infections are present at harvest or when fruits sustain superficial damage during handling and transportation. These conditions create favorable environments for fungal growth, exacerbating losses (Prusky et al., 1981a, b).

The *Alternaria* genus is characterized by the formation of dark-colored phaeodictyospores, which may occur individually or in chains. A total of 275 *Alternaria* species have been documented based on their morphology and structure (Simmons, 2007). *Alternaria* infections typically remain latent until harvest, with minimal disease progression during storage at 0 °C but significant expansion during the shelf-life period. However, high relative humidity or heavy rainfall before harvest can trigger "active infections" in wounded tissues, leading to an increased incidence of decay during storage. This necessitates shorter storage durations to prevent quality deterioration and minimize economic losses for growers.

Among *Alternaria* species, *A. alternata* has been reported as the pathogen causing black spot disease on persimmons under various climatic conditions (Prusky et al., 2001; Xiang et al., 2023). However, no studies in Pakistan have yet identified the specific *Alternaria* species responsible for diseases affecting persimmons (*Diospyros kaki* L.).

Postharvest decay symptoms have been observed on persimmon fruits in local markets in Lahore, Punjab Province. The symptoms appear as small, slightly depressed, dark brown spots (Figure 1), resembling those caused by *Alternaria* species. In severe infections, disease incidence exceeds 50%.

The present study aimed to characterize and identify the causal agent of the brown leaf spot disease affecting persimmons through morphological, molecular, and phylogenetic analyses. Five genes, *ITS*, *GAPDH*, *Calmodulin*, *Actin*, and *TEF1*, were analyzed to determine the pathogen. Moreover, a detailed study of *A. dumosa*, incorporating both morphological and molecular data, will be provided to support future taxonomic research. This foundational work on pathogen characterization represents a crucial step toward developing effective disease management strategies for persimmons.

MATERIAL AND METHODS

Collection of infected disease samples and pathogen isolation

From October to January 2021, a survey was conducted in local fruit markets in Lahore and its surrounding areas to collect persimmon fruits (*Diospyros kaki*) exhibiting necrotic black spots. The spots measured approximately 3-5 mm in size, and about 20 samples displaying similar disease symptoms were collected. On average, 30% of each fruit's surface area was affected. Diseased fruits were randomly sampled from five different markets and transported to the laboratory in sanitized polythene bags for pathogen examination.

For pathogen isolation, tissue from the necrotic black spots was excised. Small sections (approximately 3 mm^2) of the infected tissue were sterilized with sodium hypochlorite and placed onto 2% malt extract agar (MEA) medium. The plates were incubated at 25 ± 2 °C for 3-5 days in dark conditions. To obtain pure cultures, hyphal tips were sub-cultured and grown under the same conditions.

Morphological observations

Phenotypic assessments were conducted on 7-day-old fungal cultures mounted in plain lactophenol and sealed with coverslips. Both macroscopic and microscopic features were observed, including colony morphology, pigmentation, growth rate, presence of exudates, conidium size, conidial chain length, and the number, colour, and size of septa and conidia. Microscopic observations were made using a light microscope at varying magnifications. Morphological identification followed the guidelines provided in *Simmons' Manual* (2007). For morphological measurements, at least 10 replicates of conidia and conidiophores were examined across all relevant traits. Microphotography of the fungal structures was captured using a digital Olympus camera for documentation.

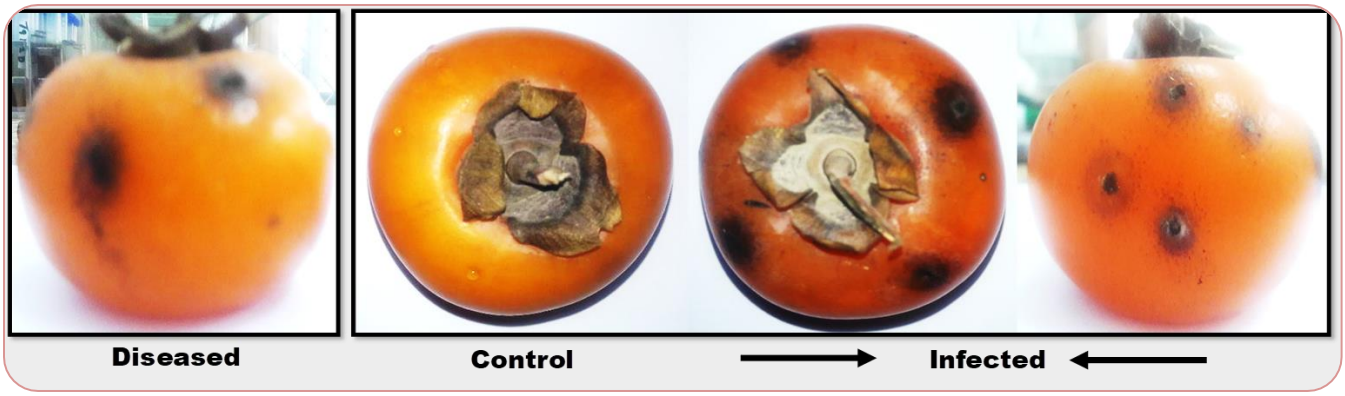

Figure 1. Persimmon fruit naturally infected with Alternaria dumosa and confirmation of Koch's postulates. Needle-wounded fungal inoculated fruits showing typical symptoms of postharvest rot.

DNA extraction, PCR amplification, and sequencing

To confirm the molecular identification of the *Alternaria* species, genomic DNA was extracted from freshly grown fungal mycelia. The mycelia were scraped using a sterile scalpel and ground into a fine powder with liquid nitrogen. Genomic DNA was extracted using the Nucleon reagent protocol described by Akhtar et al. (2014). The DNA was quantified using 1% agarose gels stained with ethidium bromide, with standards of known DNA quantities for comparison. The DNA was then diluted to the required concentration for PCR amplification.

PCR amplification employed primers ITS1/ITS4 (White et al., 1990), GAPDH (Berbee et al., 1999), ACTIN, Calmodulin (Lawrence et al., 2013), and Translation Elongation Factor (TEF) (O'Donnell et al., 1998; Carbone and Kohn, 1999) (Table 1). The PCR reaction was conducted in a thermocycler with a 25 µl reaction mixture, as described by Akhtar et al. (2014). Annealing temperatures for each primer were optimized according to the specifications listed in Table 1.

The amplification of PCR products was verified through electrophoresis on a 1% agarose gel. The resulting PCR products were then purified and subjected to sequencing for further analysis.

Phylogenetic analyses

The sequence assembly and quality assessment were initially conducted using the *Staden Package* (Staden et al., 1999), with necessary manual adjustments made where required. The raw nucleotide sequence data were analyzed using BLASTn through the National Center for Biotechnology Information (NCBI) database for preliminary sequence identification. Consensus sequences were matched against the NCBI database to facilitate initial identification and to select reference strains for phylogenetic analysis. The sequences with the highest similarity were downloaded, along with additional species used as phylogenetic references (Woudenberg et al., 2013) (Table 1).

Table 1. List of oligonucleotide primers used for the characterization of *A. dumosa* at the molecular level.

Sr. No.	Gene	Primers	Tm (℃)	Sequence (5' - 3')	Primer size (bp)
1	Actin	ACTDF1	59.9	TTCGGGTATGTGCAAGC	900-939
		ACTDR1	62	ATACCGGGGTACATGGT	
2	Calmodulin	CALDF1	62.6	AGCAAGTCTCCGAGTTCAAGG	700-763
		CALDR1	63.6	CTTCTGCATCATCAYCTGGACG	
3	Glyceraldehyde-3-phosphate dehydrogenase	Gpd1	64.5	CAACGGCTTCGGTCGCATTG	500-576
		Gpd2	62.5	GCCAAGCAGTTGGTTGTGC	
4	Internal transcribed spacer region	ITS1	64.5	TCCGTAGGTGAACCTGCGG	500-580
		ITS4	58.4	TCCTCCGCTTATTGATATGC	
5	Translation elongation factor1-alpha	TEF1	60.5	AGCCGCCGAACTCGGTAAGG	250-347
		TEF2	58.5	TCTTGGAGTCACCGGCAACG	

Multiple sequence alignments and maximum likelihood (ML) analyses were conducted using the default settings in MEGA X (Kumar et al., 1994). Multigene phylogenetic analysis was performed with Mr Bayes (Ronquist and Huelsenbeck, 2003) on XSEDE 3.2.6 via the CIPRES Science Gateway (http://www.phylo.org/). The best-fitting models for Bayesian analyses were selected using MrModeltest 2.3 within the PhyloSuite software (Nylander, 2004). Phylogenetic trees were visualized using TreeView. The obtained sequences were deposited in GenBank (Table 2). Additionally, representative isolates of the diverse fungal species identified in this study were submitted to the First Culture Bank of Pakistan.

Table 2. List of species along with their accession numbers used in this study procured by NCBI based on percentage homology.

Alternaria isolates	Culture collection No.	GenBank accession				
		ITS	GAPDH	Calmodulin	Actin	TEF
	SP001	OK447926	OL754642	OL804135	OL830275	OL962418
A. alternata		AF347031	KP124257	MG925132	JQ671702	KC584634
A. tenuissima		MT573466	AY278809	JQ646209	JQ671703	KC584693
A. arborescens		KP124401	KP124256	JQ646214	JQ671705	KC584636
A. longipes		KP124441	KP124289	JQ646198	JQ671689	KP125219
A. gaisen		KC584197	KC584116	JQ646205	JQ671699	KC584658
A. limoniasperae		FJ266476	AY562411	JQ646213	JQ671704	KC584666
A. destruens		DQ323680	MK449349	MT770803	JQ671701	
A. dumosa		KY614237	AY562410	JQ646211	JQ671695	
A. angustiovoidea		MH861939	JQ646315	JQ646203	JQ671697	JQ672465
A. lini		KP341701	JQ646308	JQ646197	JQ671688	
A. macrospora		AF229469	KC584124	JQ646243	JQ671734	KC584668
A. malvae		MK748148	JQ646314	JQ646212	JQ671696	KP125094
A. citriarbusti			JQ646322	JQ646218	JQ671709	JQ672476
A. mali		KU301746	MK399420	MH168350		MF070318
A. cinerariae		MN561359	KC584109	JQ646182	JQ671683	MN584903
A. brassicae		JQ693663	KC584102	JQ646181		KC584641
A. daucifolli		KC584193	KC584112	MH175184		
A. tomaticola		KJ651270	KJ651273	MH168355		
A. citricancri				MH107304		
A. yali inficens				MH137287		MK651809
A. martima			JQ646307	JQ646196	JQ671687	MK421633
A. brassicicola		MZ467694	LC482008			LC480214
A. porri		DQ323700	KC584132	MH175192	JQ671726	KC584679
A. sonchi		KC584220				
A. dauci		KC584192	KC584111	KY626564	JQ671732	KC584651
A. helianthiinficien		KC584200	KC584119			KC584661
A. solani		KC584217	KC584139	KJ397983	JQ671723	KC584688
A. cerealis		MH863062	JQ646321	JQ646217	JQ671708	KP125186
A. alternantherae					JQ671717	
A. alternarina		JQ646289	JQ905170	JQ646151	JQ671642	JQ672419

Confirmation of Koch's pathogenicity postulates

The pathogenicity test was conducted by inoculating healthy persimmon fruits with isolates of *Alternaria* sp. The fruits were first surface-sterilized using 2% sodium hypochlorite (NaOCl) solution and air-dried to remove excess moisture. Each fruit was then wounded with a sterile needle, and a conidial suspension (1×10^6 conidia/ml) of the *Alternaria* isolates was applied to the wounded areas. For the control treatment, distilled water was used in place of the conidial suspension. Five replicates were prepared for each treatment.

After inoculation, the fruits were placed in sterile plastic containers arranged in a completely randomized design with three replications. The containers were covered with plastic wrap to maintain high humidity. Both inoculated and control fruits were incubated at 28 °C for 7 days and monitored regularly for the development of symptoms.

RESULTS

Morphological characterization

The colony demonstrated rapid growth on malt extract agar (MEA), reaching a diameter of 5.5-6 cm within seven days. The colonies were olive-green with patches of white mycelium on the surface. The texture was cottony to woolly, with no distinct zonation observable from either the front or back of the colony (Figure 2). Sporulation was dense in areas exposed to light, characterized by short primary conidiophores, some with geniculate branching. Darker regions of the colony produced fascicles of conidiophores composed of erect hyphae. The primary conidiophores measured 4-5 µm in length and displayed geniculated conidiogenous sites.

Conidial chains branched sympodially due to the elongation of secondary conidiophores. The conidia varied in color from golden to dark brown and exhibited ovate to obclavate shapes, measuring 8-45 µm × 2-15 µm, with short conical beaks. Most conidia possessed transverse septa (1-7) and occasionally longitudinal septa (0-2), with wall ornamentation ranging from smooth to rough.

The morphological characteristics of the colonies on MEA after seven days were consistent with the description of *A. dumosa* provided by Simmons (2007).

Molecular characterization and phylogenetic analysis

The gene sequences for *ITS*, *GAPDH*, *calmodulin*, *actin*, and *TEF1* were determined to be 598, 580, 794, 900, and 250 base pairs in length, respectively (Figure 3). The *ITS* gene sequences exhibited 100% similarity with several *Alternaria* strains; however, this region alone did not permit species-level identification. In contrast, the other four genes (*GAPDH*, *calmodulin*, *actin*, and *TEF1*) showed 100% identity with *A. dumosa* strains in GenBank. The obtained sequences were submitted to GenBank, and their corresponding accession numbers are as follows: OK447926 (*ITS*), OL754642 (*GAPDH*), OL804135 (*calmodulin*), OL830275 (*actin*), and OL962418 (*TEF1*).

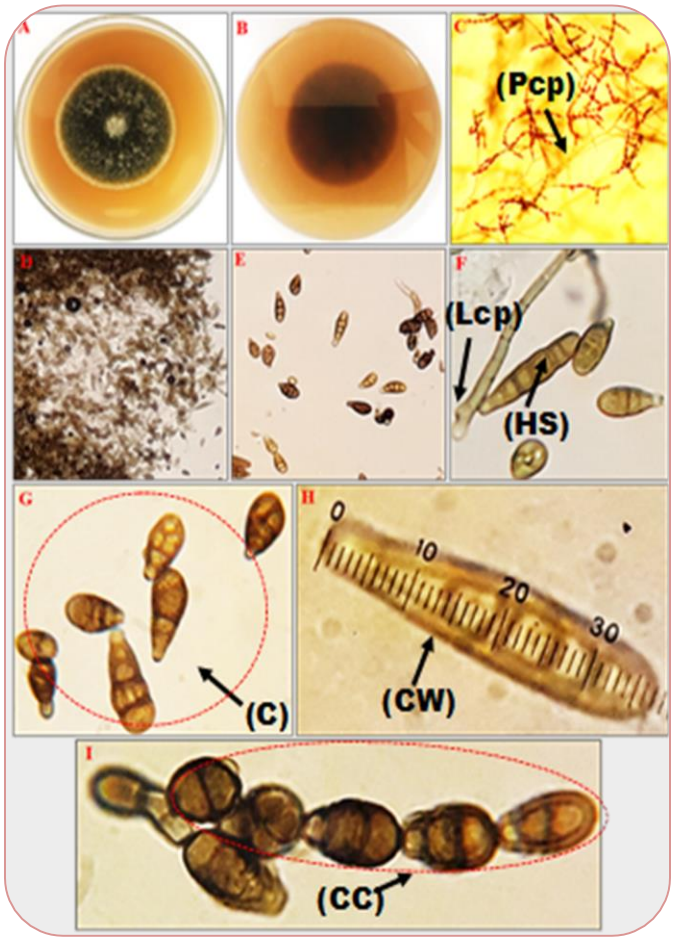

Figure 2. Morphological and cultural characteristics of *Alternaria dumosa* after 7 days of incubation on Malt Extract Agar (MEA). (A) Front view of the colony. (B) Reverse view of the colony. (C) Conidia arranged in chains observed under a stereoscope. (D) Conidia at 4× magnification. (E) Conidia at 10× magnification. (F, G) Conidia at 40× magnification. (H, I) Conidia at 100× magnification, showing hyphae and conidiophores bearing conidia.

C) = Conidia, Pcp = Primary conidiophore, Lcp = Lateral conidiophore, CC = Conidial chain, CW = Conidial Wall, HS = Horizontol septation.

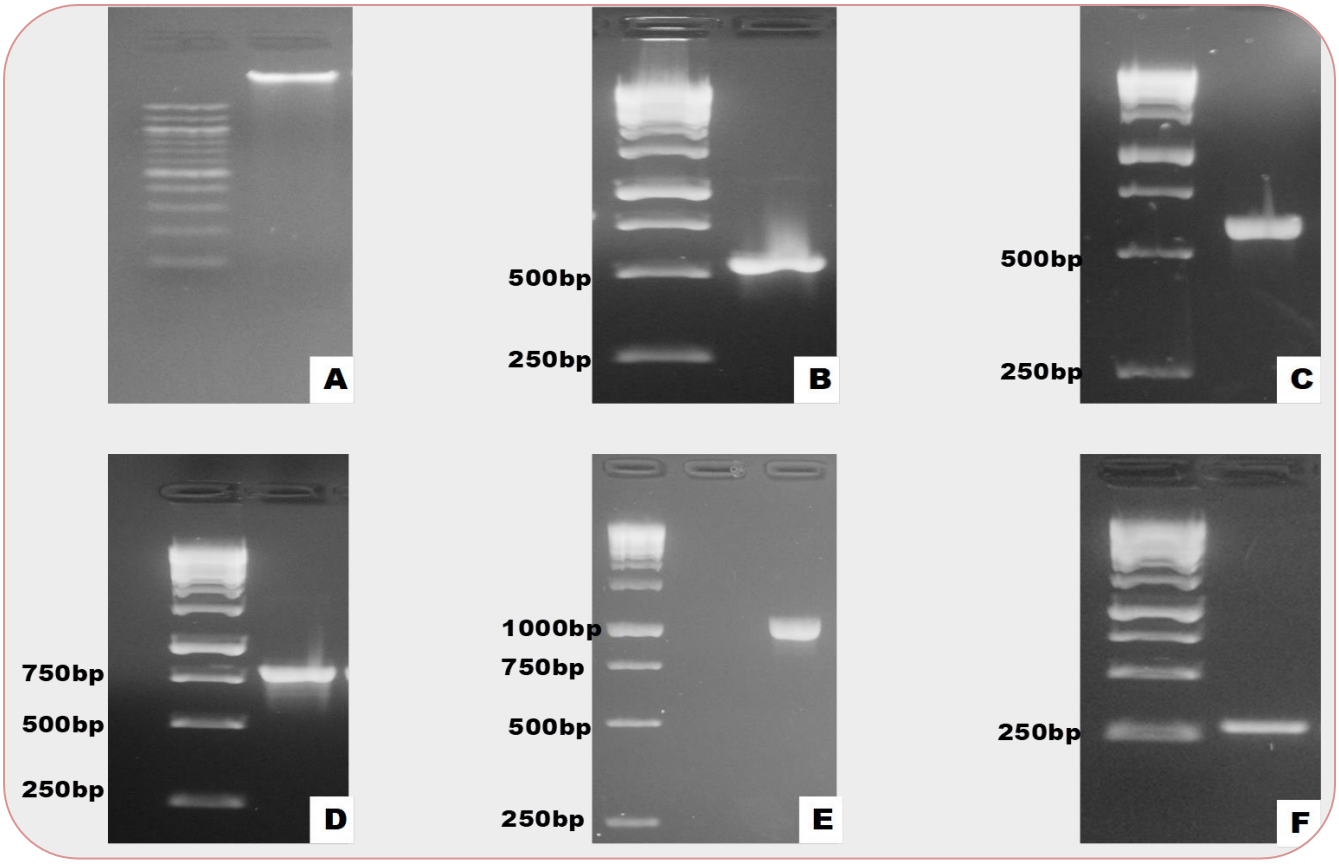

Figure 3. Agarose gel electrophoresis results. (A) Genomic DNA of *Alternaria dumosa.* (B) Amplified PCR product of the ITS region. (C) Amplified PCR product of GAPDH. (D) Amplified PCR product of Calmodulin. (E) Amplified PCR product of Actin. (F) Amplified PCR product of TEF.

Sequence differences among the isolates, along with reference isolates (Table 2), were assessed through phylogenetic analysis using maximum likelihood (ML). Phylogenetic analysis based on the *ITS* gene showed weak support values (<70%) for identifying the *A. dumosa* isolate (Figure 4A). Similarly, the phylogenetic tree based on *GAPDH* displayed weak bootstrap support for species-level identification, as the isolate clustered within a well-supported clade containing various other small-spored species (Figure 4B).

In contrast, the *calmodulin* and *actin* genes provided stronger support for identifying the isolate, as phylogenetic analysis grouped the SP001 isolate with other representative *A. dumosa* isolates (Figures 4C, 4D). The *TEF1* gene analysis also placed the isolate within the same clade as other small-spored *Alternaria* species. However, due to the lack of reference sequences for the *TEF1* gene in the NCBI database, species-level identification could not be confirmed. Consequently, the *TEF1* gene proved to be an ineffective molecular marker for species identification in this study.

Multigene sequence alignment of a combined dataset from three genes resulted in a total of 2,564 characters, including alignment gaps, with individual contributions of 570, 732, and 920 characters from *calmodulin*, *actin*, and *TEF1*, respectively. The sequences of *calmodulin*, *actin*, and *TEF1* genes were highly similar to representative isolates such as KY614237, AY562410, JQ646211, and JQ671695 of *A. dumosa*. Based on multigene phylogenetic analysis, the three isolates displayed identical sequences across all five genes and formed a strongly supported clade (100% bootstrap value) with the representative *A. dumosa* isolate available in the NCBI database (Figure 5).

Pathogenicity confirmation

To confirm pathogenicity, healthy fruits were inoculated with a conidial suspension of the isolate. The inoculated fruits developed symptoms identical to those caused by the original isolate, whereas the water-inoculated fruits remained asymptomatic (Figure 1).

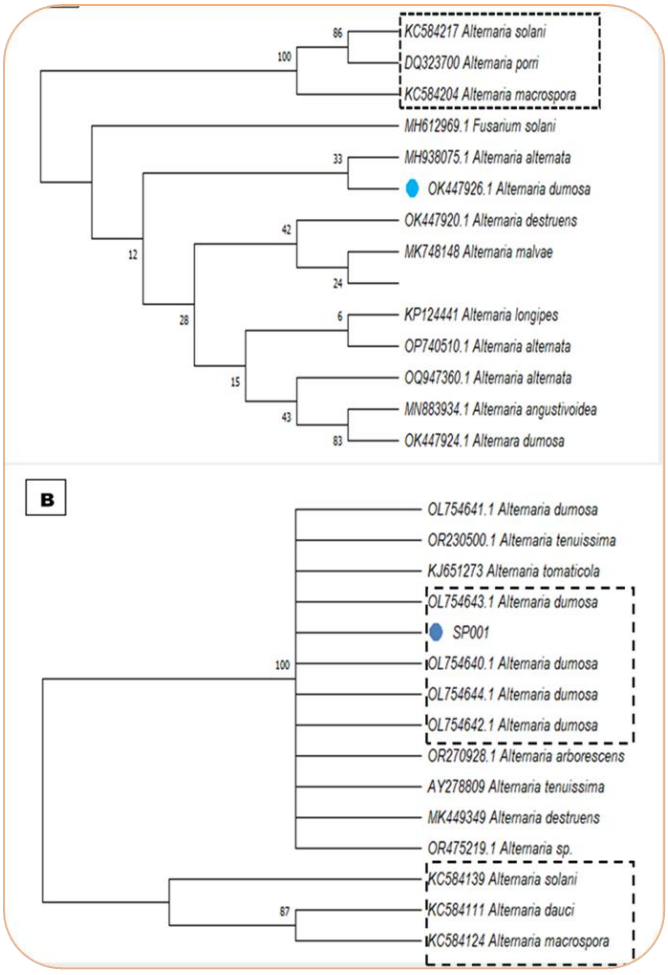

Figure 4(A). Phylogenetic analysis using the maximum likelihood method (RA×ML) performed in PhyloSuite with 1,000 bootstrap replicates. (A) Phylogenetic tree based on the ITS region. (B) Phylogenetic tree based on GAPDH.

DISCUSSION

Persimmon is a major fruit crop in Pakistan; however, fruit rot poses a significant threat to growers, resulting in substantial economic losses. In most years, *Alternaria* infections remain dormant until harvest. The disease manifests slightly during storage at 0 °C and worsens significantly during shelf life. In this study, we identified the fungal pathogen responsible for black spot disease (BSD) in persimmon fruit (*Diospyros kaki*) and characterized it using morphological, molecular, and pathogenic techniques.

The pathogen *A. dumosa* is not commonly reported, with its occurrence documented in only a few crops across various countries. For example, it has been associated with citrus in Iran, Israel, Colombia, and the United States (Simmons, 1999; Fazlikhani and Soleimani, 2013) and with potatoes in Iran (Tahery Ardestani et al., 2010). Previous studies identified *A. alternata* (Fr.) Keissl. as the primary pathogen causing BSD in persimmon fruit (Prusky et al., 1981a, b; Chohan et al., 2023; Fan et al., 2023). *A. alternata*, a closely related species, is widely reported as a major pathogen causing BSD in, particularly in regions like Japan and China (Xiang et al., 2023).

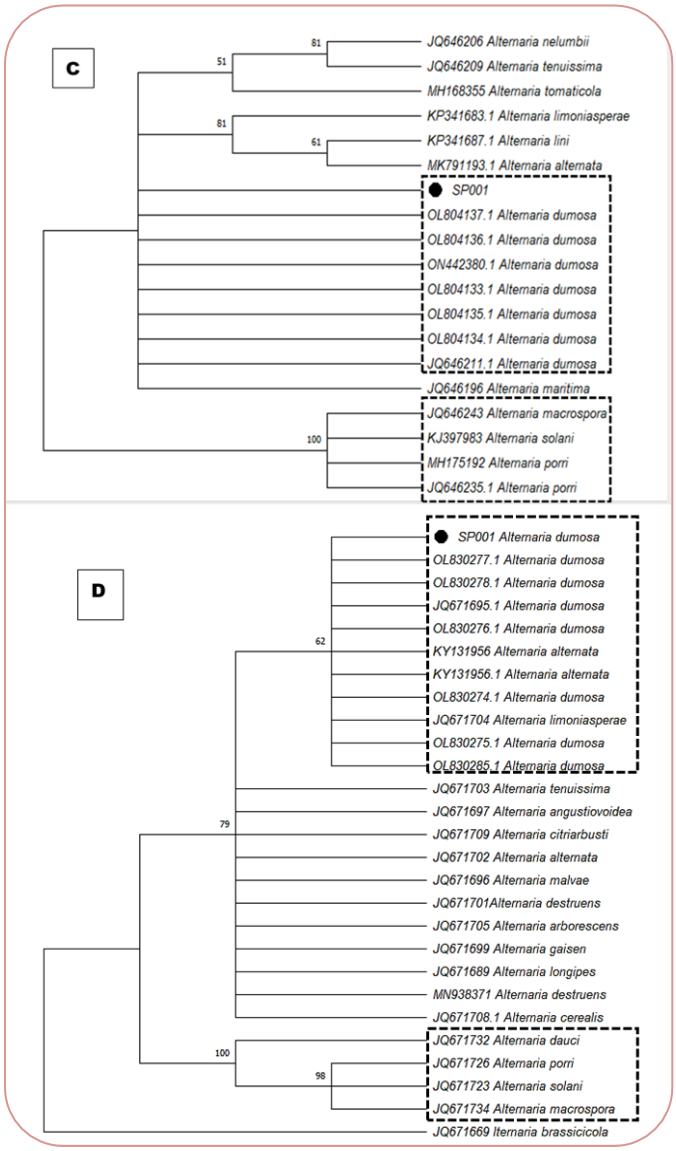

Figure 4(B). Phylogenetic analysis using the maximum likelihood method (RA×ML) was performed in PhyloSuite with 1,000 bootstrap replicates. (C) Phylogenetic tree based on Calmodulin. (D) Phylogenetic tree based on Actin. (E) Phylogenetic tree based on TEF.

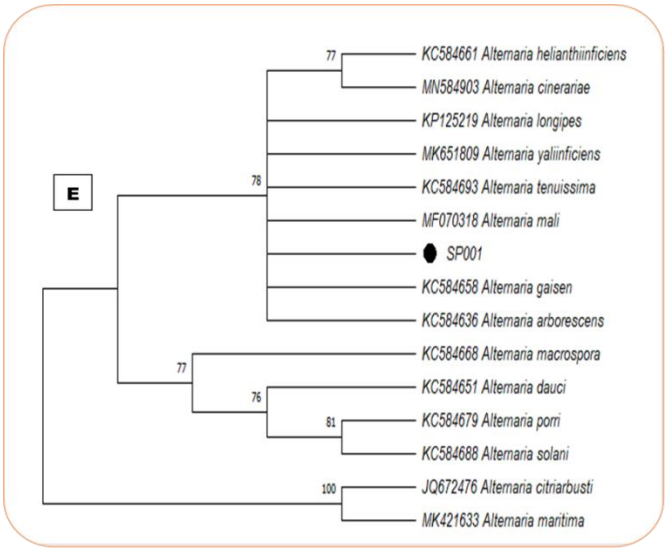

Figure 4(C). Phylogenetic analysis using the maximum likelihood method (RA×ML) was performed in PhyloSuite with 1,000 bootstrap replicates. ((E) Phylogenetic tree based on TEF.

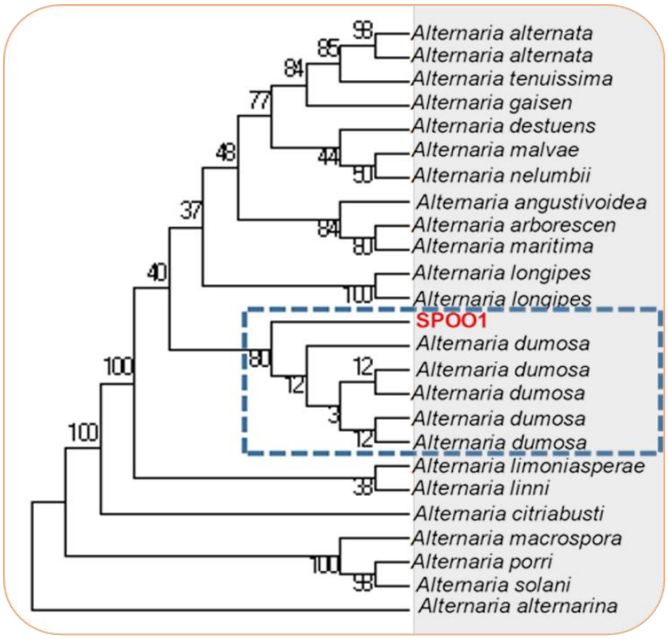

Figure 5. Multigene phylogenetic analysis estimated by three combined genes (GPDH, calmodulin and actin) sequences of 23 isolates representing the *Alternaria* species. The bootstrap support values (ML) are given at the nodes.

Similar to our findings, *A. alternata* demonstrates high adaptability to environmental stress and produces mycotoxins, such as alternariol, which compromise fruit quality and pose food safety concerns. Moreover, studies on *A. tenuissima* in Italy, Turkey, and Spain have highlighted its ability to colonize stored persimmons, leading to significant post-harvest losses (Kurt et al., 2010; Palou et al., 2012; Prencipe et al., 2023). This aligns with our observations, where *A. dumosa* exhibited similar growth capabilities under controlled conditions, suggesting its potential to impact both pre-harvest and post-harvest stages.

However, unlike *A. alternata*, *A. dumosa* has not been extensively studied regarding its secondary metabolite production. Further investigation is needed to determine its role in pathogenicity and toxin-mediated damage. Since small-spored *Alternaria* species exhibit significant morphological variation across different environments, relying solely on morphological characteristics is insufficient for precise species identification. Consequently, molecular techniques employing multiple genes are essential for accurate identification.

The results of our molecular study indicate that the highly conserved ITS region alone is insufficient for distinguishing closely related *Alternaria* species, such as *A. longipes*, *A. alternata*, and *A. tenuissima*. This finding is consistent with the observations of Shipunov et al. (2008), who reported that ITS sequences are not effective in differentiating among these species. In contrast, sequences of additional specific markers, such as GAPDH, calmodulin, actin, and TEF, have proven more reliable for identifying not only the genus *Alternaria* but also other genera, including *Botrytis*, *Fusarium*, *Ramularia*, *Aspergillus*, and *Wallemia* (Lee and Yamamoto, 2015; Zarrin et al., 2016; Trabelsi et al., 2019).

In agriculture, the accurate identification of pathogens is crucial for implementing effective management strategies. Therefore, further investigation is essential to explore the pathogenicity and host range of *A. dumosa* to develop control measures for black spot disease in persimmon fruit.

AUTHORS' CONTRIBUTIONS

SP and AM designed the study, formulated the experiments, and executed them; NA and MA collected and organized the data, analyzed the results, and wrote the manuscript; SP and MR assisted in writing the manuscript and proofreading the paper.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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