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Morphological and Molecular Characterization of *Alternaria* Species Causing Early Blight of Tomato (*Solanum lycopersicum*) in Karnataka

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Abstract

Tomato (*Solanum lycopersicum* L.) ranks as the second most important vegetable crop globally, but its production and quality are known to be largely affected by many pest and diseases. One of the major hindrances to tomato production is the fungal disease caused by *Alternaria* spp. In the current study we isolated, visually examined and characterized the fungal pathogen both morphologically and at the molecular level were carried by employing standard techniques for tissue isolation, microscopic observations and PCR methods. Under the microscope, morphological features like dark brown to blackish mycelium with prominent septations and muriform conidia with horizontal and transverse septations were observed. To delve deeper into their genetic makeup, the molecular characterization of pathogens was done by amplification, sequencing of Internal Transcribed Spacer (ITS1/ ITS4) and phylogenetic analysis. This allowed us to gain insights into the genetic identity of these pathogens. Furthermore, phylogenetic analysis shed light on the evolutionary relationships within the group of fungi we studied. This comprehensive approach provides a deeper understanding of *Alternaria* spp. in the context of tomato cultivation and offers valuable knowledge for disease management and sustainable tomato production.

Keywords : *Alternaria solani*, Internal transcribed spacer, Phylogeny, Early blight of tomato

Tomato (*Solanum lycopersicum* L.) is an annual herbaceous crop that belongs to the family, *Solanaceae*. It stands as the second most important vegetable crop grown after potato both in cultivation and consumption and cultivated year-round particularly in tropical and subtropical regions of the world. India is one of the leading countries in the production of tomato growing in an area of 8.42 lakh ha with production of 2069.43 lakh tonnes and productivity of 24.60 t ha^{-1}. The major tomato growing states in India are Andhra Pradesh, Karnataka, Maharashtra and Uttar Pradesh, contributing significantly to the country's total tomato production (Anonymous, 2023). It is the regular kitchen component of the Indian diet and is extensively consumed in various forms as a refreshing addition to salad, a base for nutrient rich juice, a key component of ketchup, a source of luscious puree or as a vital ingredient in a wide range of curries and processed food (Kang, 2016).

The high nutritional value of tomatoes is attributed to their rich content of vitamins and minerals, coupled

with its anti-cancer, antiseptic and antioxidant properties leading to a rising demand for this versatile fruit (Freeman and Reimers, 2010). Tomatoes are cultivated under a range of conditions including open and protected conditions. High humidity and warm temperatures in microclimate of tomato ecosystem not only promotes the luxuriant growth of plants but also creates favourable condition for the development of various fungal, bacterial and viral diseases (Balanchard, 1992 and Mark & Brooke, 2006). Among these, fungal diseases such as late blight, septoria leaf spot, powdery mildew, fusarium wilt and early blight caused by *Phytophthora infestans, Septoria lycopersici, Oidium neolycopersici, Fusarium oxysporum* and *Alternaria solani,* respectively are causing severe yield losses in tomato (Gleason and Edmunds, 2005).

Among the array of diseases that afflict tomato crops, the fungal pathogens stand out as significant threats, leading to substantial yield losses in tomato crops (Ellis and Martin, 1882). Early blight caused by *Alternaria* spp. is an endemic disease that presents a significant threat to tomato crop. This fungal pathogen exhibits a wide range of impact on the tomato production by infecting seedlings, leaves, petioles, stems, branches, calyx, blossom and fruits (Pandey *et al*., 2002) under favourable conditions resulting in defoliation, drying of the twigs and premature fruit drop. The consequence of this infection leads to considerable yield loss ranging from 50 to 86 per cent of the total production (Mathur and Shekhawat, 1986). Furthermore, the presence of early blight significantly affects the marketing quality of fruits. *A. solani* is the most destructive pathogen and is both airborne and soilborne in nature. Taxonomically, *A. solani* belongs to the class; euteromycetes, sub-class; Hyphomycetes, order; Moniliales and the family; *Dematiaceae* (Jones and Grout, 1986). Which causes infection in healthy plants by producing two principal toxic compounds *viz*., alternaric acid and zinniol (Pound and Stahmann, 1951). A comprehensive understanding of causal organisms with reference to morphology, cultural and physiological characters and pathogenic diversity are most important prerequisites in developing management practices. With this back ground, the current study aims to contribute to this understanding by employing morphological and molecular techniques to characterize *A. solani*, paving the way for improved disease management and crop protection.

Material and Methods

Sample Collection, Isolation and Identification

Tomato leaf and stem samples showing typical symptoms of the *Alternaria* species infection were collected from tomato plants grown in the experimental plot at the Department of Horticulture, GKVK, Bengaluru, Karnataka, India (Fig. 1). Samples were cut into small pieces and sterilized with a one per cent sodium hypochlorite solution for a minute and thoroughly rinsed with sterile distilled water to remove traces of sodium hypochlorite solution. Subsequently, the sterilized samples were aseptically transferred into sterilized petri plates containing potato dextrose agar (PDA) to facilitate fungal growth and incubated at 27 ± 1°C. After fungal growth, cultures were purified by the hyphal tip technique and pure culture was transferred onto PDA slants followed by incubation at 27 ± 1 °C under alternate light/ darkness cycles (12 h each) for three days. The resulting pure cultures were stored at 4°C for further use.

Fig. 1 : Early blight symptoms produced by *Alternaria* sp. in tomato : A) Leaf B) Stem

Pathogenicity Assay

The pathogenicity of the isolated pathogen was confirmed by atomizing the conidial spore suspension of 10mL/ plant having $3x10^6$ spores ml^{-1} prepared from ten days old culture. The spores were inoculated on to three leaves of thirty-days old seedlings of tomato hybrid Saho (Syngenta) and the spore suspension was sprayed at 3 days interval up to 12 days. The expressed symptoms were studied and re-isolation of the causal agent was done to prove the Koch's postulates.

Morphological Characterization

The morphological characteristics of isolated fungi were studied after 9 days after inoculation, by visual observation of mycelium character (colour and texture) and microscopic observations of hyphal character and spore morphologies produced by the fungus.

Molecular Characterization

DNA Extraction, PCR Amplification, Sequencing and Phylogenetic Analysis

Mycelial disc of pure culture of isolated *Alternaria* sp. was separately inoculated into the conical flask containing Potato Dextrose Broth (PDB) media under aseptic conditions and incubated for five days at 27 ± 1ºC. Ten days mycelial mat was harvested, air-dried and used for the isolation of genomic DNA by following Cetyl (hexadecyl) Trimethyl Ammonium Bromide (CTAB) method (Csaikl *et al*., 1998). Extracted DNA was stored at - 20 °C for subsequent molecular analyses. Polymerase Chain Reaction (PCR) amplification of the Internal Transcribed Spacer (ITS) region of fungi was carried out using general primers, ITS1 Forward Sequence (5'-3') CTTGGTCATTTA GAGGAAGTAA) and ITS4 (Reverse Sequence (5'-3') TCCTCCGCTTA TTGATATGC). PCR amplification was carried out in 25 µL reaction volume consisting of template DNA (100 ng/ µL), Taq buffer (2.50 µL of 10X), Mg Cl2 (1 µL of 2 mM), dNTPs mixture (2.50 µL of 1 mM), primer (5 pM of 1.50 µL), Taq DNA polymerase (1.50 µL) and sterile distilled water to make full volume of reaction mixture. PCR was performed using the Proflex PCR system (Carlsbad, California, United States). The amplification was carried out with initial denaturation of 94°C for 4 minutes followed by 35 cycles of 94°C for 60 seconds, 55°C for 45 seconds, 72°C for 90 seconds and the final extension step at 72°C for 10 minutes. Following PCR amplification, the resulted products are analyzed by on agarose gel (1 per cent w/w) electrophoresis to confirm their presence and size, often expected to be in the range of 600-800 base pairs for the ITS region (Fig. 4). Gels were stained with ethidium bromide (10 µg/mL) and viewed in a Gel documentation system to confirm the amplification of targeted DNA regions. Further, the amplified DNA products were eluted from the gel by using Qiagen gel elution kit (#Cat No. : 28706) and sequenced in both directions at Eurofins Genomics Pvt. Ltd., Bengaluru, India.

Sequencing and Phylogenetic analysis

The full-length sequence of ITS region of *Alternaria* spp. isolate was queried in NCBI database using Blast tool to find similar sequences available in the database. The sequences of different *Alternaria* spp. infecting various crops showing more homology with ITS region of the current isolate (Table 1) were retrieved from the NCBI database and aligned using BioEdit (Hall, 1999) and ClustalW (Thompson *et al*., 1994) programs. To know the evolutionary relationship of the test sequences a phylogenic analysis was performed by comparison with the sequences retrieved from the NCBI GenBank database (Table 1 and Fig. 5) using Neighbor-joining method MEGA X software with 1000 bootstrapped replications (Kumar *et al*., 2016).

Results and Discussion

Isolation of Pathogen

Infected tomato leaf samples presented with symptoms typical to *Alternaria* spp. were collected and inoculating into Petri-plates containing PDA media with the help of sterile forceps and needles under

TABLE 1

Internal Transcribed Spacer (ITS) gene nucleotide sequences of *Alternaria solani* isolates retrieved from NCBI, GenBank database for comparative analysis

Accession No.	Organism	Country	Host
OK036398.1	*Alternaria solani* isolate akosrf	India	*Solanum lycopersicum*
OK036397.1	*Alternaria solani* isolate akosrl	India	*Solanum lycopersicum*
OL865410.1	*Alternaria solani* CBS	Netherlands	*Solanum lycopersicum*
KC478609.1	*Alternaria solani* strain UP-T-10	India	*Solanum lycopersicum*
JQ625580.1	*Alternaria solani* f. sp. lycopersici	India	*Solanum lycopersicum*
LC440613.1	*Alternaria porri* AC68	Japan	*Allium fistulosum*
LC440612.1	*Alternaria porri* AC35	Japan	*Eustoma exaltatum*
LC440611.1	*Alternaria porri* AC32	Japan	*Allium fistulosum*
LC440610.1	*Alternaria porri* AC5	Japan	*Allium fistulosum*
LC440609.1	*Alternaria porri* AC16	Japan	*Allium fistulosum*
LC440608.1	*Alternaria porri* AC14	Japan	*Allium fistulosum*
JQ625586.1	*Alternaria* sp.	India	*Solanum lycopersicum*
AY154717.1	*Alternaria* sp. IA305	Iran	*Solanum lycopersicum*
LC440605.1	*Alternaria dauci* AC9	Japan	*Daucus carota*
LC440604.1	*Alternaria dauci* AC8	Japan	*Daucus carota*
AY154701.1	*Alternaria dauci*	Iran	*Daucus carota*
LC440601. 1	*Alternaria crassa*	Japan	*Datura fastuosa*
AM237287.1	*Alternaria multirostrata* amanal	Austria	*Fumana procumbens*
OR206498.1	*Alternaria tenuissima* sca057	China	*Schisandra chinensis*
OR206497.1	*Alternaria tenuissima* sca054	China	*Schisandra chinensis*
OR206499.1	*Alternaria tenuissima* sca075	China	*Schisandra chinensis*
MZ823468.1	*Alternaria alternata* b5d	China	*Solanum lycopersicum*
MZ823467.1	*Alternaria alternata* b2b	China	*Solanum lycopersicum*
MZ823466.1	*Alternaria alternata* b3c	China	*Solanum lycopersicum*
MZ823465.1	*Alternaria alternata* b2a	China	*Solanum lycopersicum*
MZ823464.1	*Alternaria alternata* b1a	China	*Solanum lycopersicum*
MZ823463.1	*Alternaria alternata* b4a	China	*Solanum lycopersicum*

aseptic conditions. The isolation procedure forms the foundational step in research endeavor, allowing for the subsequent morphological and molecular analyses that provide insights into the biology and behavior of *Alternaria* spp. and their role as fungal pathogens in tomato plants (Loganathan *et al*., 2016).

Morphological Characterization

The culture obtained from samples having typical early blight symptoms showed sparse white cottony mycelium growth with series of morphological transformations, which hold crucial implications for the identification and understanding of the pathogen responsible for this disease. Later this culture turned into whitish brown colour with appearance of fluffy, smooth and flat mycelium. Finally, the mycelium turned into brownish black colour (Fig. 2). Furthermore, a closer examination of the culture revealed its ability to produce separate conidia, each borne on simple conidiophores. These conidia were

Fig. 2 : a) *Alternaria* sp. grown on PDA media
b) Muriform conidia with septate mycelium

found to possess distinct morphological features, characterized by their elongated, muriform shape and a colour spectrum spanning from light to dark brown. Their ellipsoidal, flexuous structure with pale beaks and produced singly, which contributed to their unique profile. Importantly, these conidia exhibited 5-8 horizontal septa with 0-2 vertical septa on the branched and septate mycelium. The dark brown mycelium with the muriform conidia was the typical morphological feature of the genus, *Alternaria* (Fig. 2). This comprehensive morphological analysis of the culture provides valuable insights into the life cycle and characteristics of the pathogen responsible for early blight, which is often caused by *Alternaria* sp. In our investigation, the conidial dimensions of the pathogen isolated from tomato plants showing typical symptoms of early blight were in conformity with the standard initial descriptions (75-350 x 20-30 µm) by Ellis and Martin (1882) and later which was reaffirmed by Rao in 1969. These findings are consistent with previous research conducted by Varma *et al*. (2006), Yadav and Pathak (2011), Kumar *et al*. (2016), Nguyen *et al*. (2013) and Srikanth, *et al*., (2023). The conidial dimensions reported in our study, in line with these earlier references, contributing to the validation of the pathogen's identity and provide essential supporting evidence for its classification as *Alternaria* species. Such congruence with established standards and the corroboration by previous research reinforce the robustness of our observations and the reliability of our morphological characterizations.

Pathogenicity Assay

In our pathogenicity test, the isolated fungus exhibited a distinctive pattern of symptom production on inoculated tomato leaves which appear in disease progression of early blight. Initially, small yellowish lesions became apparent within 3 to 4 days after inoculation (DAI), which later turn to light brown followed by dark brown spots on the leaves (6 to 8 DAI). The spots gradually increased in size, became circular to oval with or without concentric rings, with clear chlorotic halo and attained 0.3-2.2 cm in diameter by 18 DAI. In addition, disintegration of central necrotic area of some lesions and shot hole formation was noticed with respect to the untreated check plants which were maintained without any inoculation of fungal spore or mycelial suspension (Fig. 3). The culture reisolated and purified from these artificially infected leaves demonstrated identical characteristics to the original culture, confirming the pathogenicity of the isolated fungus. The same infection process and symptoms were observed in the studies of Devi (2014) and Biswas (2016). These findings collectively contribute to our understanding of early blight highlighting the importance of pathogenicity testing in the study of plant diseases.

Fig. 3 : a) Pathogenicity test showing symptomatic expression of *Alternaria* infected tomato leaf.
b) Leaf spot with concentric rings symptoms.

Molecular Characterization and Phylogenetic Analysis

The DNA isolated from early blight causing pathogen isolated from leaf plant sample collected from Department of Horticulture, GKVK, Bengaluru, Karnataka, India was subjected to PCR amplification using universal primers ITS1/ITS4. The process resulted in amplification of ~650 bp fragment (Fig. 4). The ITS is the common conserved region which is used for the differentiation of the fungi at species level, which corresponds to ITS rRNA region of the fungal species. Subsequently, the PCR product was sequenced and the nucleotide sequence of ITS region of *Alternaria* spp. isolate in the current study was compared with the representative nucleotide sequences of ITS regions of selected fungal species from the GenBank database. The results revealed that the fungi isolated in the current study shared the maximum nucleotide identity of 98.7 per cent with *Alternaria solani* ACrCrL isolate infecting tomato reported from India, Following, the next closest identities were observed with *Alternaria solani* f. sp. *lycopersici* (LC440605), *Alternaria solani* UP-T-10 (KC478609), *Alternaria solani JZJ* (OP019832), *Alternaria solani* alt NL03003 (OL865410) and *Alternaria solani* AKoSrL (OK036397) which showed the 98 per cent identity. In contrast, the lowest identity of 97.20 per cent was observed to the *Alternaria* sp. IA305 (AY154717) isolate reported from the Iran (Table 2).

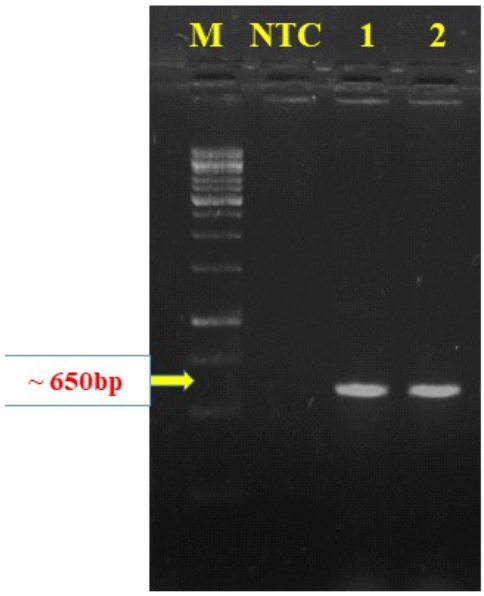

Fig. 4 : Ethidium bromide stained agarose gel showing PCR amplicon of internal transcribed spacer (ITS) region M: 1kb ladder; NTC: Non template control; 1 and 2. *Alternaria* sp.

Phylogenetic analysis findings provided further evidence that, there was a five different clustering *viz*., *Alternaria alternata, Alternaria pori, Alternaria solani, Alternaria dauci* and *Alternaria tenuisimma.* Among them *Alternaria alternata* showed the major clustering and the fungi isolated in the current study, showed the close clustering with *Alternaria solani* and revealing a more distant relationship with *Alternaria alternata* isolates (Fig. 5). This molecular characterization and phylogenetic analysis provide a deeper understanding of the genetic identity and evolutionary context of the early blight pathogen. It underscores the significance of using molecular tools to elucidate the genetic relationships and taxonomy of fungal pathogens and their implications for plant diseases.

The research underscores the importance of integrating both morphological and molecular approaches in the study of plant diseases. The results of the current study clearly indicated the existence of *A. solani* causing early blight in tomato and this species is one among the widely distributed in and around the tomato growing areas of Bengaluru and Kolar regions of Karnataka, India. The ability to accurately identify and characterize the pathogen responsible for early blight is crucial for the development of effective disease management strategies. These insights are invaluable for agricultural practices, contributing to the sustainability and productivity of tomato crops. This study not only advances understanding of early blight but also serves as a foundation for future research and efforts aimed at mitigating the impact of this fungal pathogen on tomato cultivation.

TABLE 2

Per cent nucleotide sequence identities of ITS region of *Alternaria solani* with other selected *Alternaria* spp sequences retrieved from NCBI GenBank.

Accession No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
OK036379	100																											
JQ625586	98.7	100																										
LC440605	97.9	98.4																										
LC440604	97.9	98.4	100	100																								
AY154717	97.7	97.9	98	98	100																							
AY154701	97.9	98.4	100	100	98	100																						
JQ625580	98.9	99.8	98.6	98.6	98	98.6	100																					
OP019832	99.1	99.6	98.4	98.4	97.9	98.4	99.8	100																				
KC478609	99.1	99.6	98.4	98.4	97.9	98.4	99.8	100	100																			
OL865410	99.1	99.6	98.4	98.4	97.9	98.4	99.8	100	100	100																		
OK036398	99.1	99.6	98.4	98.4	97.9	98.4	99.8	100	100	100	100																	
OK036397	99.1	99.6	98.4	98.4	97.9	98.4	99.8	100	100	100	100	100																
LC440613	98.9	99.4	98.2	98.2	97.7	98.2	99.6	99.8	99.8	99.8	99.8	99.8	100															
LC440612	98.9	99.4	98.2	98.2	97.7	98.2	99.6	99.8	99.8	99.8	99.8	99.8	100	100														
LC440611	98.9	99.4	98.2	98.2	97.7	98.2	99.6	99.8	99.8	99.8	99.8	99.8	100	100	100													
LC440610	98.9	99.4	98.2	98.2	97.7	98.2	99.6	99.8	99.8	99.8	99.8	99.8	100	100	100	100												
GKVK	98.6	98	97.5	97.5	97.2	97.5	97.9	98	98	98	98	98	97.9	97.9	98	97.9	100											
LC440609	98.9	99.4	98.2	98.2	97.7	98.2	99.6	99.8	99.8	99.8	99.8	99.8	100	100	100	100	97.9	100										
LC440608	98.9	99.4	98.2	98.2	97.7	98.2	99.6	99.8	99.8	99.8	99.8	99.8	100	100	100	100	97.9	100	100									
MZ823468	93.9	93.7	94	94	94.6	94	93.5	93.4	93.4	93.4	93.4	93.4	93.4	93.4	93	93.4	95.3	93.4	93.4	100								
MZ823467	93.9	93.7	94	94	94.6	94	93.5	93.4	93.4	93.4	93.4	93.4	93.4	93.4	93	93.4	95.3	93.4	93.4	100	100							
MZ823466	93.9	93.7	94	94	94.6	94	93.5	93.4	93.4	93.4	93.4	93.4	93.4	93.4	93	93.4	95.3	93.4	93.4	100	100	100						
MZ823465	93.9	93.7	94	94	94.6	94	93.5	93.4	93.4	93.4	93.4	93.4	93.4	93.4	93	93.4	95.3	93.4	93.4	100	100	100	100					
MZ823464	93.9	93.7	94	94	94.6	94	93.5	93.4	93.4	93.4	93.4	93.4	93.4	93.4	93	93.4	95.3	93.4	93.4	100	100	100	100	100				
MZ823463	93.9	93.7	94	94	94.6	94	93.5	93.4	93.4	93.4	93.4	93.4	93.4	93.4	93	93.4	95.3	93.4	93.4	100	100	100	100	100	100			
OR206499	93.7	93.5	94	94	94.4	94	93.4	93.2	93.2	93.2	93.2	93.2	93.2	93.2	93	93.2	95.1	93.2	93.2	99.8	99.8	99.8	99.8	99.8	99.8	100		
OR206498	93.7	93.5	94	94	94.4	94	93.4	93.2	93.2	93.2	93.2	93.2	93.2	93.2	93	93.2	95.1	93.2	93.2	99.8	99.8	99.8	99.8	99.8	99.8	100	100	
OR206497	93.7	93.5	94	94	94.4	94	93.4	93.2	93.2	93.2	93.2	93.2	93.2	93.2	93	93.2	95.1	93.2	93.2	99.8	99.8	99.8	99.8	99.8	99.8	100	100	100

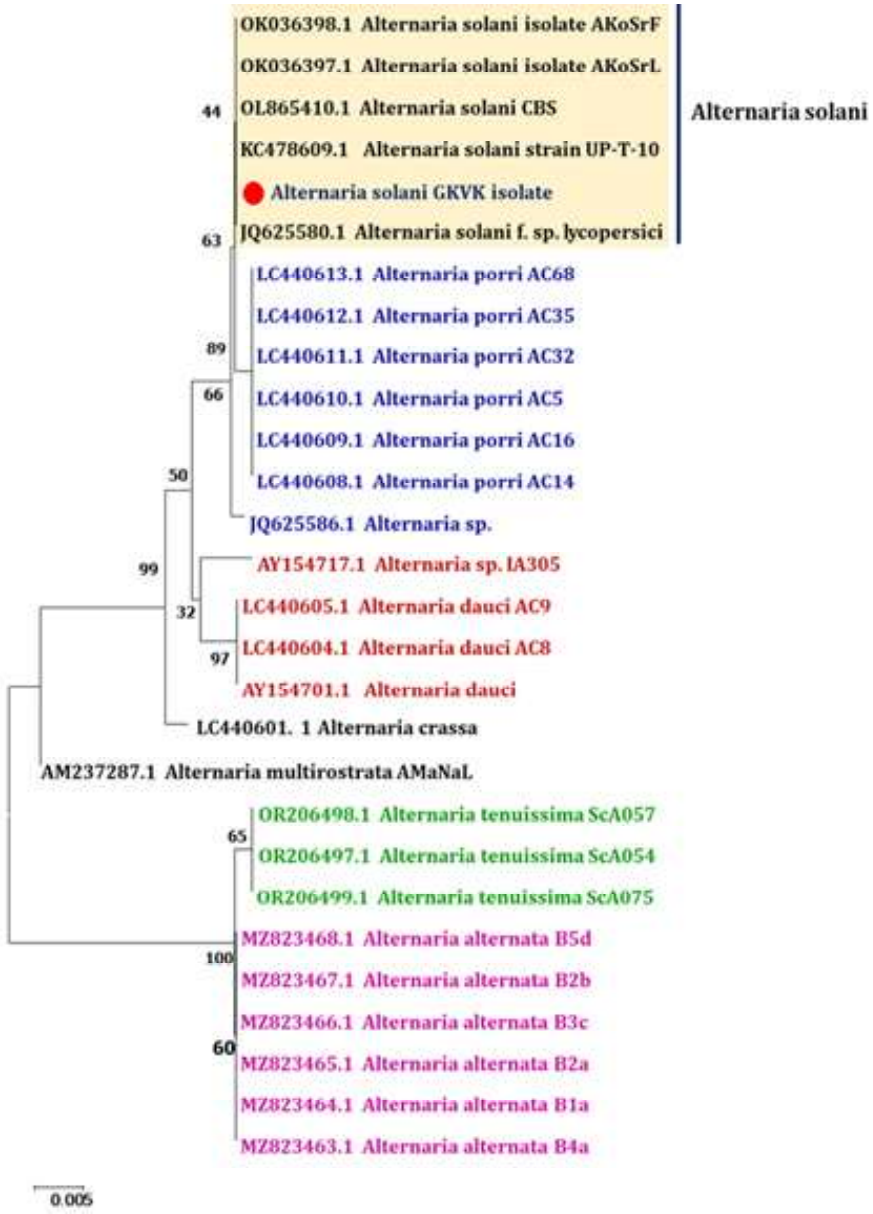

Fig. 5 : Phylogenetic tree constructed from nucleotide sequences of Internal Transcribed Spacer (ITS) region of Alternaria solani isolate infecting tomato with sequences of related species retrieved from NCBI GenBank using Neighbor-joining method available in MEGA X

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