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INTEGRATED MORPHOLOGICAL, PATHOGENIC, AND MOLECULAR CHARACTERIZATION OF *ALTERNARIA ALTERNATA* ASSOCIATED WITH EARLY BLIGHT OF TOMATO (*SOLANUM LYCOPERSICUM* L.)

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ABSTRACT

The present investigation focuses on the isolation and comprehensive characterization of *Alternaria alternata*, the causal agent of early blight in tomato (*Solanum lycopersicum* L.). Infected leaf tissues were processed using standard tissue segment techniques, and pure cultures were obtained on potato dextrose agar (PDA). Morphologically, the isolates exhibited characteristic greyish colonies with concentric zonation and conidia arranged in chains, with both transverse and longitudinal septa. Pathogenicity assays performed on tomato seedlings confirmed Koch's postulates, establishing the virulence of the isolates. Molecular characterization was conducted by amplifying the ITS region using universal primers ITS1 and ITS4. Sequencing results were analyzed through BLAST, confirming the identity of the isolates as *A. alternata*, and sequence data were submitted to NCBI GenBank under accession number ON845591. Phylogenetic analysis using the UPGMA method revealed high genetic similarity with previously reported *A. alternata* isolates. This study contributes to the accurate identification and molecular diagnostics of early blight pathogens, facilitating targeted disease management strategies.

***Keywords*:** *Alternaria alternata*, Early blight, Tomato, ITS sequencing, Morphological identification, Pathogenicity, Phylogenetic analysis.

Introduction

Tomato (*Solanum lycopersicum* L.) is one of the most popular vegetable crop in the world, shares a coveted position in India. It is one of the important food and cash crop for many low-income farmers in the tropical countries so also regarded as poor man's apple. Among the vegetable crops tomato ranks the second position in world and rank first among the processing crops. It is a native of tropical America and is cultivated in about 130 different countries. Brazil, China, Cuba, Egypt, Indonesia, Russia, Spain *etc.,* are the leading producers of tomato.

Tomato is one of the versatile vegetables with wide usage in Indian culinary tradition. It is used as a fresh vegetable and also variety of processed products such as juice, ketchup, sauce, canned fruits, puree, paste, *etc*.

Tomato is a rich source of vitamins and minerals mainly rich in vitamin C and minerals especially phosphorus, potassium and calcium. Nutritive value

per 100 g of edible part is carbohydrate 3.9 g, protein 0.9 g and fat 0.2 g. Besides, it is a good source of "lycopene" pigment which is largely responsible for the red colour of fruit. It is also containing antioxidants which include carotenoids such as β-carotene and phenolic compounds such as flavonoids and hydroxycinnamic acid derivatives (Borguini and Torres, 2009).

Characterization of pathogen based on virulence is mandatory to develop appropriate management practices. Identification of early blight pathogen is generally based on conidial morphology under specific media, temperature, relative humidity and light (Kumar *et al.,* 2008). Genetic identification is progressively used to identify the pathogens (Bridge *et al.,* 2004) and internal transcribed spacer (ITS) rRNA has been successfully employed to identify the fungal pathogens at species level (Bowmann *et al.,* 2007). In the present study, *Alternaria* early blight infected tomato samples collected from research field were used to characterize *Alternaria* spp. based on cultural characteristics, pathogenic potentiality and ITS region.

Material and Methods

Characterization based on Culture and Spore Morphology

Isolation, purification and maintenance of pathogens:

Laboratory experiments were conducted in the Department of Plant Pathology, Horticultural Research Station, Venakataramannagudem. Infected tomato plants showing disease symptoms were collected from Centre of Excellence and used for isolation and pathogenicity test. Isolation of the pathogen was made under aseptic conditions by tissue segment method (Aneja, 2003) from the samples showing typical symptoms Tissues bits of infected sample along with a portion of adjacent healthy tissue was (3 to 5 mm in length) surface sterilized with 1 % sodium hypochlorite solution for three minutes and subsequently washed thrice with sterile distilled water to remove the traces of sodium hypochlorite. The tissues were spread on the filter paper until complete drying. Then, they were placed on potato dextrose agar (PDA) medium supplemented with streptomycin sulphate to prevent bacterial contamination. The inoculated plates were incubated in BOD at 25 ± 2^{o} C for 5 days. The culture was identified based on morphological characters (Subramanyam 1970; Suresh *et al.,* 2011). The pathogen was purified separately by transferring the tip of the mycelia into fresh media plates and maintained on media slants which were stored at 4^{0} C as stock cultures for further studies.

Maintenance of the pure culture

The fungal pathogen was sub-cultured on PDA slants and incubated at 27 ± 1^{o} C for ten days and such slants were preserved in refrigerator at 4^{0} C and revived once in 30 days.

Identification of the pathogen

These studies were undertaken to confirm the identity of the pathogens. Specific structure of pathogens likes mycelium and spores were observed under microscope (10x) and (40x).

Pathogenicity test of isolated organisms:

Pathogenicity is the capability of the pathogen to cause disease. The pathogenicity of the isolated pathogens was tested and established according to koch's postulates. Pathogenicity test of the isolates was conducted according to Jasnic *et al.* (2005). Pathogenicity of the isolated pathogen was tested on tomato seedlings in artificially infected soil mixture made of sterilized soil and pathogen suspension (1×10^{6} spores per milliliter). In case of foliar disease-causing pathogens, the isolated pathogens were sprayed/ inoculated by following methods of inoculation. In case of soil borne diseases inoculum suspension was prepared by pouring 50 ml autoclaved water in petri plate containing 10 days old culture, stirring the culture with sterilized glass rod. Control plants were sown in soil with sterile distilled water. Plants were incubated at 22-25^{0} C for 14 days. The pathogen was re-isolated from plant showing typical symptoms and plating on PDA medium.

Pathogenicity test of early blight of tomato was conducted on healthy plants of tomato. Seeds were sown in the polybags contained sterilized soil. Spore suspension was prepared for artificial inoculation by flooding the fungal culture grown on PDA with sterilized distilled water and later suspension concentration was adjusted to 1×10^{6} spores per milliliter by using haemocytometer. When seedlings were at 5-7 true leaf stage the seedlings were inoculated by spraying the spore suspension on the leaves using a fine atomizer until the leaves were completely wet. Equal volume of sterile distilled water was used in case of control. After inoculation, seedlings were incubated at 25°-30°C and covered with polythene bags to maintain 90-100 per cent relative humidity to promote infection and lesion expansion. The observations were taken for the appearance and development of symptoms.

Molecular characterization

Molecular studies were carried out at central lab, College of Horticulture, Anantharajupeta. Molecular characterization of isolate begins with DNA extraction (Gul *et al.,* 2017) from the collected isolate. The gene regions were amplified by PCR using the specific primers given below. Then samples were sequenced and compared with the gene bank data base through the BLAST programme.

Preparation of stock solutions for molecular work

Tris HCl (1 M)

12.114 g of HCl (Mol.wt. 121.4) was dissolved in 70 ml of distilled water. The pH was adjusted to 8 by adding concentrated HCl to the contents and then the volume was made up to 100 ml by adding distilled water.

EDTA (0.5 M)

18.62 g of EDTA (Mol.wt. 372.44) was dissolved in 70 ml of distilled water and stirred vigorously using magnetic stirrer. Then the pH was adjusted to 8 by adding NaOH base and the volume was made up to 100 ml by adding distilled water.

NaCl (3 M)

17.53 g of NaCl (Mol.wt.58.44) was dissolved in 70 ml of distilled water and the volume was made up to 100 ml by adding distilled water.

Chloroform: isoamyl alcohol (24:1) (100ml)

Chloroform (96 ml) and isoamyl alcohol (4 ml) were mixed properly using vortex mixture for 2 min. The prepared solution was used as 24:1 chloroform: isoamyl alcohol.

Sodium acetate (3 M)

24.60 g of Sodium acetate (Mol.wt. 82.03) was dissolved in 70 ml of distilled water. After complete dissolving, the solution was made upto 100 ml by adding distilled water.

Ethanol (70 %)

Absolute ethanol (70 ml) was added to 30 ml of distilled water to prepare 100 ml of 70% ethanol.

Composition and preparation of TBE buffer:

10x TBE buffer

Tris buffer	-	54 g
Boric acid	-	27.5 g
EDTA	-	4.65 g
Distilled water	-	500 ml

Tris, boric acid and EDTA of required quantity were dissolved in 200 ml of deionized water. The volume was made up to 1000 ml by adding deionized water to form 10X TBE buffer. Undissolved white clumps were dissolved completely with the aid of a magnetic stirrer. This can used as stock solution, 1X TBE buffer was made by taking 100 ml of stock solution and dissolving in 900 ml of deionized water and the same was used as running buffer for gel electrophoresis.

Preparation of loading dye

1% Bromophenol blue	-	200µ
Glycerol	-	400µl
10% SDS	-	60µl
0.5M EDTA	-	50µl
10X TBE	-	60µl
Distilled water	-	30µl

All the above chemicals of required quantity were taken in to a 2ml effendorf tube, and mixed well using a vortexe mixture for 1minute, this dye was used as loading dye for gel electrophoresis.

Procedure

For the isolation of DNA, initially the fungal culture was grown for 5 days in conical flasks containing potato dextrose broth (PDB) for getting a mycelial mat. This was done by transferring the mycelial discs of the fungus from 7 days old culture in to PDB. The fungal mycelial mat was obtained by filtering through the filter paper followed by drying between two layers of the filter paper. Then 0.4 g of mycelial mat of test pathogen was grounded in mortar and pestle using liquid nitrogen to form a fine powder. The powdered form of mycelium was transferred into 1.5 ml tubes after addition of CTAB buffer, allowed to incubate at 65^0 C for 1 hour in water bath, and centrifuged at 10,000 rpm for 10 minutes. Then one ml of liquid supernatant was added with equal volume of chloroform: Isoamyl alcohol (24:1) and was centrifuged for 10 minutes at 10,000 rpm at room temperature. After that upper layer was taken into separate eppendorf tube and was added with equal volume of ice-cold isopropanol and 0.1 volume of 3M sodium acetate (pH 5.2) and incubated over night at -20^0 C. On the next day after incubation, it was centrifuged at 12,000 rpm for 15 minutes then pellet was taken and 70% ethanol was added to the pellet, centrifuged at 10,000 rpm for 5 minutes and finally the pellet was dissolved in Milli Q water.

PCR amplification and sequencing

The isolated DNA was amplified with universal primers ITS 1 (TCCGTAGGTGAACCTGCGG) and ITS 4 (TCCTCCGCTTATTGATATGC) in PCR (White *et al.* 1990). The initial step was done at 94^0 C for 5 minutes, denaturation at 94^0 C for 60 seconds, annealing at 55^0 C for one minute, extension step at 72^0 C for 1.5 minutes and final extension at 72^0 C for 15 minutes and repeated for 34 cycles followed by cooling at 4^0 C for 30 seconds.

Quantification of Genomic DNA

The total obtained genomic DNA concentration was measured using U.V. Spectrophotometer Nano drop (ND-1000). Blank was kept against millique water. For estimating the concentration of DNA, the optical density was measured at 260nm. The concentration of DNA was related to the optical density by the following equation. The optical density (O.D) was recorded at 280nm to calculate the ratio OD260/OD280. The ratio at a value of 1.8 indicating best DNA preparation. The ratio below 1.8 indicates the presence of proteins in the preparation and a value of above 1.8 indicates that the sample has more DNA.

Loading of agarose gel

Gel plates of 13 x 14 cm dimensions were washed thoroughly with cleaning solution followed by distilled water and dried. The plates were sealed with cellophane tape at the two open sides. Then ethidium bromide (1.5µg/µl) was added to the gel at hand tolerable heat. Then the solution was poured in the gel plate along with the comb and the solution was allowed to polymerize.

Loading and gel electrophoresis

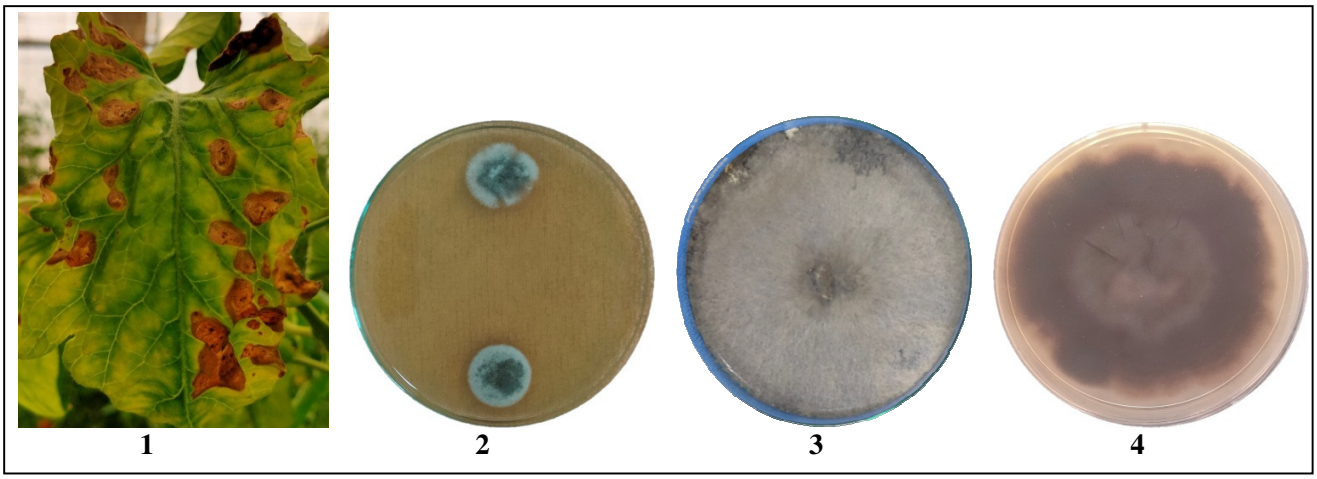
After polymerization, the inserted comb was gently removed from the gel. The gel plate was placed in horizontal electrophoretic apparatus and the tank was filled with 1X TBE buffer. With the help of micropipettes, the samples were loaded in the wells. Loading dye of 5µl was added with the help of micropipettes into each DNA sample and mixed well. After loading, the electrophoretic unit was connected with power pack with a regulated electric power supply of 100V. At the end of gel run, the gel was carefully removed, the gel image was viewed in U.V. transilluminator gel doc (Alpha Innotech Multi image light cabinet filter positions) and stored in gel documentation system. Gene Ruler100-bp plus DNA ladder (© 2012 Thermo Fisher Scientific Inc.) was used as a molecular weight marker.

Results and Discussion

Cultural characterization

Identification of the pathogen

The culture of the fungal colony was initially white, cottony with profuse aerial mycelium which gradually turned grey colour. Aged culture appeared completely greyish with aerial mycelium and distinct concentric rings was formed on medium. Conidiophores were short to long, simple or branched arising singly. Conidiophores were golden to brown coloured with 2-9 transverse and 0-2 longitudinal septa. Conidia were born in long chains (6-11) on conidiophores, they were thick walled, beaked and brown in colour. Based on the characters of the colony and morphological characters of conidiophores and conidia the fungus was identified as *Alternaria alternata* (Plate 2).



1. Leaf showing typical early blight symptoms
2. Growth of mycelium three days after inoculation
3. Isolated pathogen
4. Lower side of the petri plate

Plate 1: Isolation of *Alternaria alternata* causing early blight of tomato (Tissue segment method).

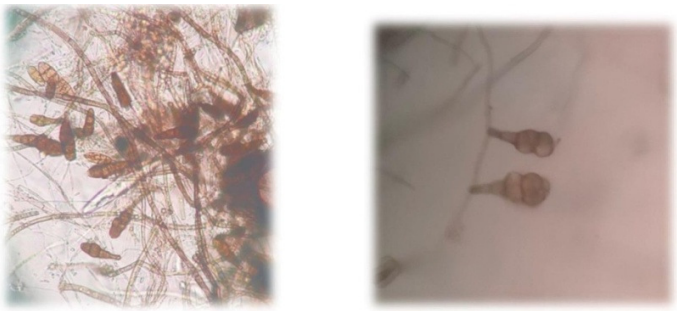
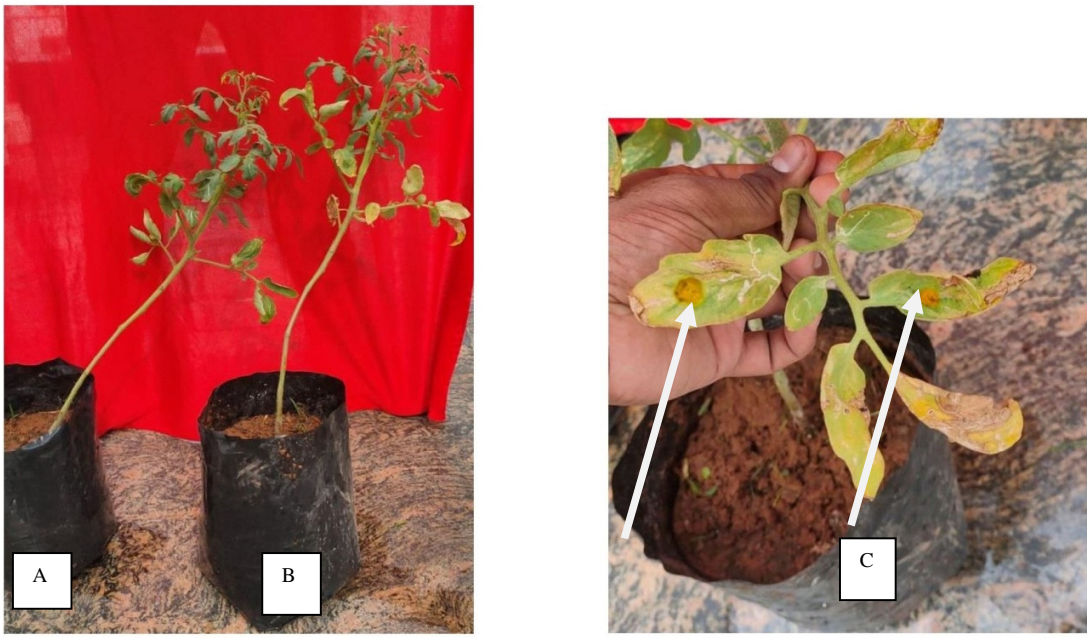


Plate 2: Photograph showing conidia of *Alternaria alternata* causing early blight of tomato.



A. Control
B. Inoculated plant
C. Formation of concentric circles on tomato leaves during pathogenicity

Plate 3: Proving pathogenicity of the *Alternaria alternata* causing early blight of tomato.

Pathogenicity test

The pathogenicity test is necessary to confirm the pathogenic nature of the organism. Symptoms of inoculated pathogens showing typical disease symptoms 5-7 days after inoculation on tomato leaves. Thereafter, the pathogen was re-isolated from the infected lesions and compared with original culture

PCR amplification and phylogenetic analysis

Genomic DNA (18s Ribosomal DNA) was isolated by CTAB method and subjected to agarose gel electrophoresis to observe the DNA output. Clear band was observed indicating the presence of the DNA. Purified DNA was stored at - 20ºC for further analysis. Genomic DNA was amplified using universal primers ITS1(5' TCCGTAGGTGAACCTGCG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC 3') by performing 35 cycles of initial denaturation at 94°C for 5min, denaturation at 94°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 1.5 min, final extension at 72°C for 5 min. Amplified PCR products were subjected to agarose gel electrophoresis and gel was pictured by transilluminator in Gel doc unit. Amplified ITS gene sequences of the fungal isolates yielded a band at in between 500-550 base pair.

PCR product sequencing

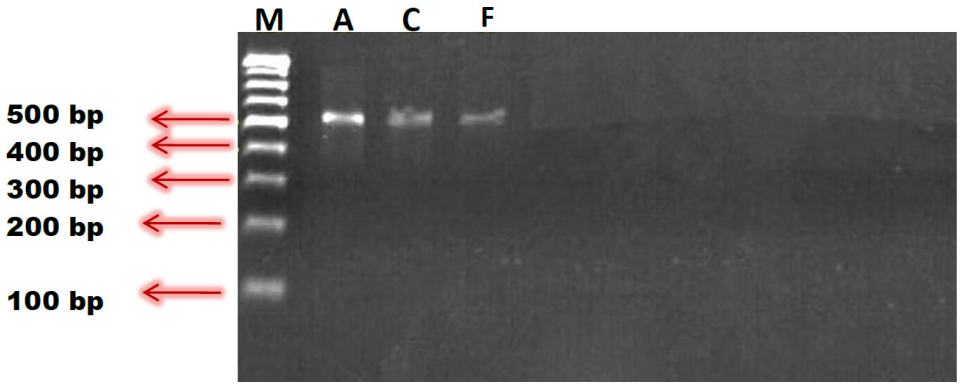
Further process of sequencing PCR product (20µl) was sent to Eurofins Genomics, Bengaluru. Homology searches of rDNA sequences of the isolate were performed using online nucleotide BLAST program through National Centre for Biotechnological Information (NCBI). The results obtained through BLAST analysis revealed that the sequences of isolates showed similarity with *Alternaria alternata.*

Isolated fungal sequences were deposited in NCBI genebank as *Alternaria alternata* RG1 internal transcribed spacer 1, partial sequence; 5.8s ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and larger subunit ribosomal RNA gene, partial sequence. Accession numbers *Alternaria alternata* (ON845591) was given by NCBI

Construction of phylogenetic tree using UPGMA (Unweighted pair group method with arithmetic mean) method.

Sequence data was aligned and analysed for finding the closet homologous isolates. Phylogenetic tree was plotted to understand the relation between the test isolate with various other isolates.

The evolutionary history was inferred using the UPGMA method (Sneath and Sokal, 1973). The optimal tree is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (50 replicates) was shown next to the branches. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al.*, 2004) and are in the units of the number of base substitutions per site. This analysis involved 29 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 1215 positions in the final dataset.



Lane M: Molecular weight marker of 100 base pair DNA ladder
Lane A: Isolate of *A. alternata*
Lane C: Isolate of C. *gloeosporioides*
Lane F: Isolate of *F. solani*

Plate 4: PCR amplification for ITS region of *A.alternata* with ITS-1 and ITS-4 ribosomal DNA primers.

```
>YSRHU_TOMATO_AA
AAATATGAAGGCGGGCTGGAATCTCTCGGGGTT
ACAGCCTTGCTGAATTATTCACCCTTGTCTTTT
GCGTACTTCTTGTTTCCTTGGTGGGTTCGCCCA
CCACTAGGACAAACATAAACCTTTTGTAATTGC
AATCAGCGTCAGTAACAAATTAATAATTACAAC
TTTCAACAACGGATCTCTTGGTTCTGGCATCGA
TGAAGAACGCAGCGAAATGCGATAAGTAGTGTG
AATTGCAGAATTCAGTGAATCATCGAATCTTTG
AACGCACATTGCGCCCTTTGGTATTCCAAAGGG
CATGCCTGTTCGAGCGTCATTTGTACCCTCAAG
CTTTGCTTGGTGTTGGGCGTCTTGTCTCTAGCT
TTGCTGGAGACTCGCCTTAAAGTAATTGGCAGC
CGGCCTACTGGTTTCGGAACCCAACACAAGTCG
CACTCTCTATCAGCAAAGGTCTAGCATCCATTA
AGCCTTTTTTCCAACTTTTGACCTCGGATCAGG
TAGGGATACCCGCTGAACTTAAGCATATCATAA
CCGGGAGGAAAG
```

Plate 5 : Sequencing of *Alternaria alternata*

```
LOCUS       YSRHU_TOMATO_AA          540 bp    DNA     linear   PLN 26-JUN-2022
DEFINITION  Alternaria alternata isolate YSRHU_TOMATO_AA.
ACCESSION   ON845591
VERSION
KEYWORDS    .
SOURCE      Alternaria alternata
  ORGANISM  Alternaria alternata
            Eukaryota; Fungi; Dikarya; Ascomycota; Pezizomycotina;
            Dothideomycetes; Pleosporomycetidae; Pleosporales; Pleosporineae;
            Pleosporaceae; Alternaria; Alternaria sect. Alternaria; Alternaria
            alternata complex.
REFERENCE   1  (bases 1 to 540)
  AUTHORS   Sairam,K., Gopal,K., Srinivasulu,B., Ruth,C., Ramadevi,P.,
            Ravindrababu,M. and Padmaja,V.
  TITLE     Direct Submission
  JOURNAL   Submitted (26-JUN-2022) Plant Pathology, Dr. Y.S.R. HORTICULTURAL
            UNIVERSITY, Venkataramannagudem, Tadepalligudem, Andhra Pradesh
            534101, India
COMMENT     ##Assembly-Data-START##
            Sequencing Technology :: Sanger dideoxy sequencing
            ##Assembly-Data-END##
FEATURES             Location/Qualifiers
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                     /organism="Alternaria alternata"
                     /mol_type="genomic DNA"
                     /isolate="YSRHU_TOMATO_AA"
                     /isolation_source="Leaf"
                     /host="Solanum lycopersicum (Tomato)"
                     /db_xref="taxon:5599"
                     /country="India: AndhraPradesh"
                     /collection_date="10-Apr-2022"
                     /collected_by="Sairam Kudupudi"
                     /note="PCR primers=fwd name: ITS-1, rev name: ITS-4"
```

Plate 6 : Accession number ON845591

The study indicated that *Alternaria alternata* exist as a virulent form to cause early blight disease in tomato as documented earlier Kumar *et al.* (2008); Naik *et al.* (2010); Murugan *et al.* (2014); Bhatt *et al.* (2000); Xie *et al.* (2012), Loganathan *et al.* (2014), Isabela *et al.* (2019), Raghupathi *et al.* (2020), Kusaba and Tsuge (1995), Pryor and Gilbertson (2000), Wang *et al.* (2001) and Pryor and Michailides (2002).

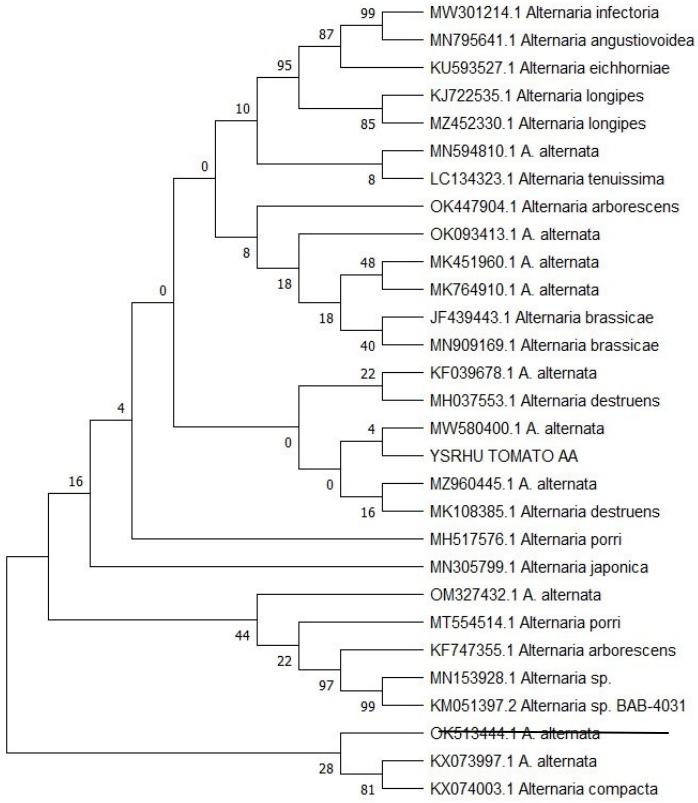

Fig. 1: Dendrogram of *Alternaria alternata* using UPGMA method

Conclusion

Genomic DNA of the *Alternaria alternata* isolated by using CTAB method. For identification of the pathogens at molecular level, the genomic DNA was amplified with universal primers (Forward) ITS 1 and (Reverse) ITS 4. From the results obtained, the genomic DNA was amplified at 500-600 base pair. Sequence analysis revealed that the sequences of isolates showed similarity with *Alternaria alternat.*. Isolated fungal sequences were deposited in NCBI genebank and accession numbers of *Alternaria alternata* (ON845591) given by NCBI.

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