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Isolation, Pathogenic, Morphological and Molecular characterization of *Alternaria solani* causing Post-harvest Rot of Tomato Fruits

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ABSTRACT: *Alternaria solani*, which is the causal organism of post-harvest rot of tomato, is one the devastating pathogen causing severe yield losses worldwide. Since it causes around 60 to 80 per cent yield losses in tomato fruits, study of this pathogen becomes necessary at post-harvest level. This study focuses on identification of morphological, pathogenic and molecular variability among the various test isolates of *A. solani* isolated from tomato fruits *in vitro*. Infected samples were collected from markets around Coimbatore district. The results concluded that, the eight isolates of *A. solani* exhibits morphological variability based on colour and colony character and pathogenic variability based on severity of infection caused on tomato fruits. The molecular characterizations of these isolates were carried out using universal primers ITS 1 and 4 and sequenced. The phylogenetic analysis was performed by neighbour joining method. This study focuses on the isolation, pathogenic, morphological and molecular characterization of *Alternaria solani* isolates causing post-harvest rot of tomato fruits.

Keywords: Tomato, post-harvest rot, *Alternaria solani*, morphology, molecular, pathogenic.

INTRODUCTION

Tomato *(Solanum lycopersicum* L.) is a major contributor to the fruit and vegetable diet of humans. India stands second in the production of tomato next to China (FAOSTAT, 2017). It is cultivated in essentially all areas of the country either in fields or in protected culture. It is grown widely throughout the country, either in field or in greenhouses in cooler climates. Tomato is grown over an area of 29.08 thousand hectares with an annual production of 887.08 MT during 2017-18 (Anonymous, 2019) in Tamilnadu. Tomato fruit compositionally, has unique nutritional and phytochemical profile. Vitamin C, Vitamin A, potassium, fibre and antioxidants like lycopene are present naturally in tomatoes. Among the pathogens causing post-harvest diseases in tomato, *Alternaria solani* is an important pathogen. *Alternaria solani* belongs to the phylum Ascomycota, class Dothideomycetes, order Pleosporales and to family Pleosporaceae (Simmons, 2007). The post-harvest rot caused by *A.solani* is a serious threat to the profitable cultivation of tomato (*Solanum lycopersicum* L.) which on the other hand reduces the quality and quantity of tomato fruits drastically. The symptoms of the disease are characterized by brown to dark brown coloured necrotic spots. Under humid condition, these spots are progressed upwards and coalesced to produce the concentric zone on the leaves, appearing like the bull's eye. In fruits, lesions are observed at the stem-end which is dark, leathery and sunken with target board like appearance (Agrios, 2005). In case of severe infection, the pathogen leads to defoliation, drying off of twigs and premature fruit drop causing 50% to 86% losses in fruit yield. Although *A. solani* fungus is cosmopolitan in nature requires several specific compounds for their growth. A wide range of media can favour the isolation of *A. solani* fungus which supports the radial growth, dry weight growth and sporulation of fungus. Compositions of various media can also influence the characters like colony morphology of *A.solani*. Morphological characterization is one of the classical approach to distinguish fungal species, which is one of the main requisite of fungal taxonomy. Cultural, morphological and molecular variability was also documented by several workers (Kaul and Saxena 1998; Yunhui *et al*., 1994; Babu *et al*., 2000; Ahmad, 2002; Naik *et al*., 2010; Rahmatzai *et al*., 2016). Identification of *A. solani* species has been done traditionally based on the morphological characters like the growth habit of the pathogen, conidia, ascogonia, antheridia and rate of growth in culture media. Since, these morphological characteristics could be influenced by culture conditions and epidemiological conditions many species are very similar when compared under this category, which makes the use of molecular methods to differentiate species becomes necessary. The use of molecular methods for identification of *A.*

solani species began more than a decade ago. Understanding the variability of the pathogen will help in developing effective management strategies. Therefore genetic variation analysis within the pathogen populations is very helpful in understanding coevolution of host-pathogen, epidemiology and developing strategies for resistance management (Leung *et al.,* 1993). Use of ITS regions seems to be the most popular choice of many researchers working with detection of this pathogen. This study focuses on the isolation of the pathogen from infected tomato fruit, morphological characterization for identification of the pathogen and molecular characterization using ITS rDNA.

MATERIALS AND METHODS

Isolation and identification of pathogen. *A. solani* infected samples were collected from different markets around Coimbatore. The collected samples were used for the isolation of pathogen *A. solani*. Infected tissues of tomato fruits were cut into small pieces and it is surface sterilized by dipping in 0.1% mercuric chloride for 30 seconds and then given three subsequent washings with sterile distilled water (Kator *et al*., 2018). The sterile pieces were placed on petri plates containing Potato Dextrose Agar (PDA) medium by half plate technique and the plates were incubated at 27± 2°C. After a week of incubation using single hyphal tip method the culture was transferred to plates containing fresh Potato dextrose agar (PDA) medium and incubated at 27± 2°C. Further, the pure culture of each isolates was maintained separately on PDA slants in glass test tubes and stored in refrigerator for future studies (Koley *et al*., 2015).

Pathogenicity study. To study the pathogenic ability of *A.solani* isolates, tomato fruits of hybrid Saago were used. Unripe, green tomato fruits were collected and surface sterilized with 70 % ethanol. Injuries were made on the fruit with the help of sterilized needle. Mycelial disc of the isolates obtained from 7 days old culture grown on PDA medium, were inoculated on the fruits and these were placed in boxes containing moist cotton. In order to maintain humidity, the boxes were covered with polythene sheets. Three replications were maintained for each isolate and monitored regularly. Furthermore, to study the virulence potential variability of each isolate, the disease incidence and severity were continuously measured. Observation in each 14 days interval was recorded, using 0-5 disease scale (Pandey *et al.,* 2003). Disease incidence and per cent disease index (PDI) for each isolate were calculated as follows:

Per cent disease incidence =

$$\frac{\text{Number of diseased Fruits} \times 100}{\text{Total Number of Fruits observed}}$$

Percent disease index (PDI) =

$$\frac{\text{Sum of all rating} \times 100}{\text{Total number of observations} \times \text{maximum rating grade}}$$

Morphological characterization. All the eight isolates *A. solani* were isolated aseptically on the PDA plates, incubated at 27±2°C for a week and cultural characteristics were observed which are colony colour/pigmentation, colony morphology, colony diameter and mycelial growth is observed 15 days after incubation. Morphological characters like mycelium and conidial characters were observed at 1000X using phase contrast microscope (Nikam *et al*., 2015).

Molecular characterization. The *A. solani* fungal culture were grown in conical flask containing 50 ml of potato dextrose broth for about 10 days and maintained at 25±1°C in BOD. Genomic DNA was extracted by pure fungal culture with slight modification of CTAB protocol (Manicom *et al.,* 1987). Mycelium was extracted by filtration through Whatmann No. 1 filter paper and washed exhaustively with distilled water. For each isolate 5 g of fresh mycelial mat was dried on sterile blotter paper and grounded with liquid N_2 to make fine powder. The one gram of grinded fungal culture was taken in eppendorf tube and 15 ml of Cetyl trimethyl ammonium bromide CTAB (DNA extraction buffer) was added in each tube separately and incubated at 65°C in water bath for about 30 min with intermittent shaking. The mixture was centrifuged at 13,000 rpm/min for 15 min at 4°C to pellet the mycelium. Supernatant was taken in a fresh Oakridge tube and an equal volume of phenol: chloroform: isoamylalcohol (25:24:1) was added with 2 – 3 min slow inversion. The mixture was again centrifuged at 13,000 rpm/min for 15 min at 4°C. The aqueous supernatant was taken in a fresh tube and added 0.6 volume isopropanol and was incubated at – 20°C overnight. After overnight incubation, it was again centrifuged at 13,000 rpm/min for 20 min at 4^0C temperature. The supernatant was discarded and the pellet was washed with 70% ethanol. The pellet was dissolved in 500 µl of TE buffer and used for PCR amplification.

PCR Amplification. *A. solani* cultures were identified molecularly using the conserved ribosomal Internal Transcribed Spacers (ITS) region. Using the universal primer pairs ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'), amplified the ITS regions between the small nuclear 18S rDNA and the larger nuclear 28S rDNA, including 5.8S rDNA. PCR was carried out in a final volume of 25 µl, containing 10mM of oligonucleotide primer (1 µl), 20mM of each of the four deoxynucleotide triphosphates (0.75 µl), 25mM $MgCl_2$ (1.0 µl), 0,35 µl of Taq DNA polymerase, 10 × Assay buffer (2.5 µl) and 1.0 µl template DNA (60 ng/µl) (Virendra Kumar *et al.,* 2008). All PCR reactions were carried out using Mastercycler® Nexus X2 PCR cycler (MA, USA) with the following parameters: an initial denaturation step at 95°C for 2 mins, 40 cycles of amplification with denaturation at 95°C for 1 min, annealing at 58°C for 1 min and 72°C for 1 min for extension with final extension 72°C for 10 min (Nikam *et al*., 2015). The electrophoresis was carried out on 1.0 per cent agarose gels. The Bio-Rad Gel Doc EZ Imaging System was used to view the PCR (Biotium, Hayward, CA).

Sequencing of ITS region and analysis. The PCR products were eluted and sequenced further at Biokart India Pvt Ltd, Bangalore, India. Partial nucleotide sequences of rDNA ITS region of isolates of *A. solani*

were downloaded from NCBI database (www.ncbi.info). The program Basic Local Alignment Search Tool-Nucleotide or BLASTn server, was used to edit and align the ITS sequences and the similarity between strains of 18S rRNA gene sequences were calculated using Clustal W (Hall.,1999 and Thompson *et al.*, 1997).The aligned sequnces were deposited in the GenBank database.

Phylogenetic Analysis. The phylogenetic tree was constructed in Mega XI software (Kumar *et al.*, 2018) with 1000 bootstrap replications using the Neighbour Joining approach based on the Tamura 3-parameter model (Tamura 1992). The out group used in this phylogenetic tree analyses was *Sclerotium rolfsii* and the reference sequence for 18S rRNA was obtained from GenBank data.

RESULTS AND DISCUSSION

Isolation of pathogen. From the infected samples collected from different markets around Coimbatore, eight isolates of *A. solani* was obtained. Isolation was carried out on PDA medium since it is the best medium to support the pathogen growth according to the study carried out by Koley *et al*. (2015). The results revealed that these eight isolates of *A. solani* exhibited differences in cultural characteristics like colony colour/pigmentation, colony morphology, colony diameter and mycelial growth which is shown in Table 1. The eight *A. solani* isolates were isolated using hyphal tip method. The eight isolates were designated as T AS1, T AS2, T AS3, T AS4, T AS5, T AS6, T AS7 and T AS8.

Morphological characterization. Mycelial growth of T AS1 was recorded as 45.55 mm, T AS 2 was 86.87 mm, T AS 3 was recorded at 50.78 mm, T AS 4 has recorded mycelial growth of 51.45 mm, T AS 5 has recorded mycelial growth of 49.56 mm, T AS 6 was 82.67 mm, T AS 7 has mycelial growth of 89.23mm and T AS 8 was recorded at 87.11 mm. Maximum mycelial growth was recorded by T AS 7(89.23 mm). The least growth was noticed in T AS1. The eight isolates of *A. solani* exhibited different colony characters on PDA or Potato dextrose medium (Table 1). The colony characters were circular, raised colonies with concentric zonation were the characteristic feature of T AS 1, T AS 4 and T AS 7. The colony characters of T AS 2 were irregular, flattened colonies without concentric zonation. In contrast, the T AS 3 have irregular, flattened with concentric zonation. T AS 5 has colonies that are raised, irregular, with concentric zonation. Irregular colonies which are flat and had no concentric zonation were seen in T AS 6. T AS 8 had colonies that are circular, flat and has concentric zones. Conidia of virulent isolate T AS 7 is cylindrical, elongated with 3 to 4 transverse septations and 1 to 2 vertical sepatations which is shown in Fig. 1.

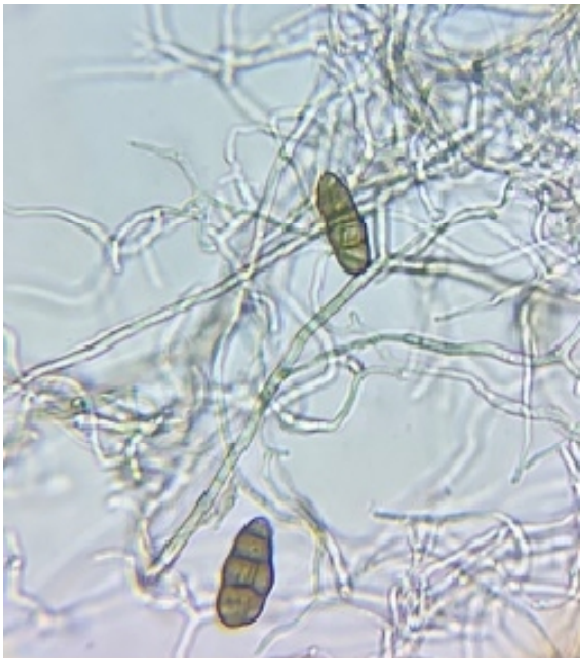
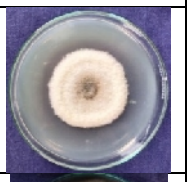
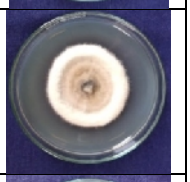
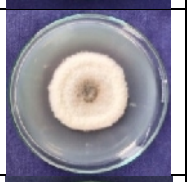
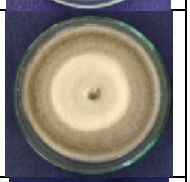
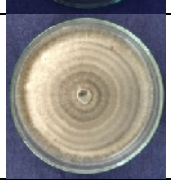


Fig. 1. Conidia of *Alternaria solani.*

Table 1: Morphological characters of *A. solani* isolates isolated from tomato fruit.

Sr. No.	Isolate	Location of sample collection	Colony diameter (mm)	Colony colour/pigmentation	Colony characters	Appearance on PDA medium
1	T AS 1	Uzhavar santhai	45.55[h]	White	Circular, concentric zonation and raised colonies	
2	T AS 2	Saibaba colony	86.87[c]	Dull grey	Irregular, no concentric zonation and flattened colonies	
3	T AS 3	Thondamuthur	75.78[e]	Greyish white	Irregular, concentric zonation and flattened colonies	
4	T AS 4	Vadavalli	51.45[f]	Creamish white	Circular, concentric zonation and raised colonies	
5	T AS 5	Gandhipuram	49.56[g]	Dull black	Irregular, concentric zonation and raised colonies	
6	T AS 6	Pazhamudhir nilayam	82.67[d]	Black	Irregular, no concentric zonation and flattened colonies	
7	T AS 7	Uzhavar santhai	89.23[a]	Blackish white	Circular, concentric zonation and flattened colonies	
8	T AS 8	Ukkadam	87.11[b]	Greyish black	Circular, concentric zonation and flattened colonies	

*Mean of three replications
In a column, means followed by a common letter is not significantly different at 5% level by DMRT

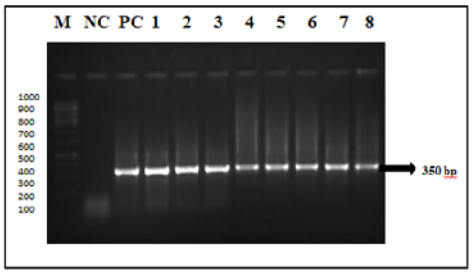
Pathogenicity study. Pathogenicity test which was conducted *in vitro* on fully matured tomato fruits of Saago hybrid revealed that after 5 days of inoculation typical symptoms were developed on all tomato fruits inoculated with *A. solani*, whereas the untreated control fruits did not exhibit any symptoms. Koch's postulates were satisfied by constant re-isolation of *A. solani* from pathogen inoculated fruits. The virulency of these isolates was tested 14 days after inoculation. These isolates obtained from several locations exhibited differences in their virulence. All the isolates of *A. solani* successfully established infection on tomato fruits, but the degree of infection was varied. Among the test isolates T AS7 was more virulent with maximum level of disease incidence (80%) and disease severity (73.4%) (Table 2).

Table 2: Disease incidence and disease severity of *A.solani* isolates in tomato fruits.

Sr. No.	Isolate	*Percent Disease Incidence	*Percent Disease Index (PDI)
1.	T AS 1	60.0[d]	48.7[c]
2.	T AS 2	73.3[b]	62.0[b]
3.	T AS 3	26.6[h]	20.5[h]
4.	T AS 4	53.4[e]	37.2[e]
5.	T AS 5	53.3[f]	45.8[d]
6.	T AS 6	46.8[g]	28.9[f]
7.	T AS 7	80.0[a]	73.4[a]
8.	T AS 8	66.7[c]	26.5[g]

* Mean of three replications
In a column, means followed by a common letter is not significantly different at 5% level by DMRT

Molecular characterization and Phylogenetic analyses. The Internal Transcribed Spacer (ITS) regions (ITS1 and ITS4) and 5.8S gene area of 18S rDNA were amplified with the primers ITS1 and ITS4 to validate the initial identification and identity the clear taxonomic position. All eight isolates were amplified with 350 base pairs which has confirmed it as *A. solani*. Similarly Mohammadi and Bahramikia (2019) obtained the amplicon size of 350 bp in *Alternaria* isolates. The amplification were identical with prior identity and the amplified 18S-rDNA (ITS 1 and ITS 4) region was purified individually and sequenced by sangar dideoxy sequencing in NCBI (Fig. 2). The sequence of ITS regions were shown 98% sequence homology with GenBank sequences with BLASTn analysis. The sequence results obtained from the BLASTn analysis were submitted in NCBI GenBank, and the isolate T AS7 was assigned with the accession number as ON745167. The phylogenetic tree has formed five clades. The isolate T AS 7 used in this study comes under cluster 3. Several subclusters were formed in this cluster. The 89% of similarity was attained with other *A. solani* strains collected from gene bank.



Lane
M – 100 bp ladder, L1 – Negative control,
L2 Positive control, L3 T AS 1, L4 T AS 2,
L5 – T AS 3, L6 – T AS 4, L7 – T AS 5,
L8 T AS 6, L9 T AS 7, L10 T AS 8

Fig. 2. ITS fragments amplification of *Alternaria solani* isolates using universal primers ITS 1 and 4.

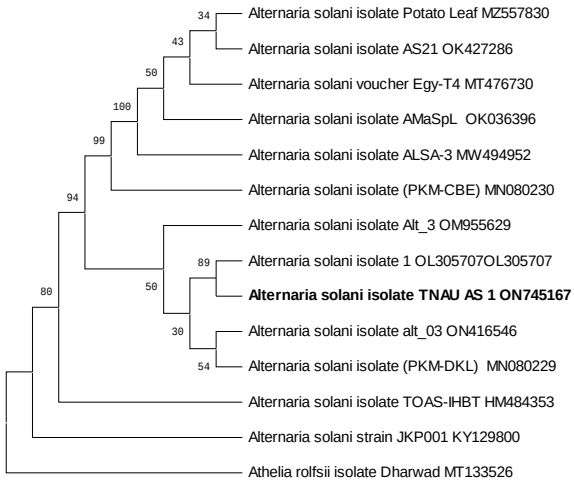


Fig. 3. Phylogenic tree generated from 18s rDNA sequence of *A. solani* using Neighbour likelihood analysis in MEGA11

CONCLUSION

Based on the findings, it has been inferred that *A.solani* isolates obtained from different locations in the Coimbatore region may differ in terms of pathogenicity, morphology, and molecular makeup.

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Conflict of Interest. None.

REFERENCES

Agrios, G. N. (2005). Plant Pathology. 5th edition. Elsevier Academic Press, New York. pp.665.

Ahmad, S. (2002). Conidial morphology of *A. solani* and its variation in tomato. *Ann. Agri. Res.*, *23*(3): 514-515

Anonymous (2019). Horticultural statistics at a glance. Horticultural statistics Division, Department of Agriculture, Cooperation & Farmers Welfare, Ministry of agriculture & Farmers Welfare, Government of India. pp. 1-437.

Babu, S., Seetharaman, K., Nandakumar, R., and Johnson, I. (2000). Variability in cultural characteristics of tomato leaf blight pathogen. *Plant Disease Research*, *15*(1), 121-121.

FAOSTAT (2017). Available online: http://faostat.fao.org/site/339/default.aspx.

Hall, T. A. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. In *Nucleic acids symposium series* (Vol. 41, No. 41, pp. 95-98). [London]: Information Retrieval Ltd., c1979-c2000

Kator, L., Iheanacho, A. C., and Aloho, K. P. (2018). Isolation, identification and pathogenicity of fungal organisms causing postharvest spoilage of tomato fruits during storage. *Annual Research & Review in Biology*, 1-7.

Kaul, A. K., and Saxena, H. K. (1988). Physiologic specialization in *Alternaria solani* causing early blight of potato I. cultural and physiologic variability. *Indian J. Mycol. Pl. Path.*, *18*: 128-132

Koley, S., and Mahapatra, S. S. (2015). Evaluation of culture media for growth characteristics of *Alternaria solani*, causing early blight of tomato. *J. Plant Path. Microbiol. S*, *1*.

Kumar, S., Stecher, G., Li, M., Knyaz, C., and Tamura, K. (2018). MEGA X: molecular evolutionary genetics analysis across computing platforms. *Molecular biology and evolution*, *35*(6), 1547.

Kumar, V., Haldar, S., Pandey, K. K., Singh, R. P., Singh, A. K., and Singh, P. C. (2008). Cultural, morphological, pathogenic and molecular variability amongst tomato isolates of *Alternaria solani* in India. *World Journal of Microbiology and Biotechnology*, *24*(7), 1003-1009.

Leung, H., Nelson, R. J. and Leach, J. E. (1993). Population structure of plant pathogenic fungi and bacteria. *Adv. Plant Pathol.10*: 157205.

Mohammadi, A., and Bahramikia, S. (2019). Molecular identification and genetic variation of *Alternaria* species isolated from tomatoes using ITS1 sequencing and inter simple sequence repeat methods. *Current Medical Mycology*, *5*(2), 1.

Naik, M. K., Prasad, Y., Bhat, K. V., and Rani, D. (2010). Morphological, physiological, pathogenic and molecular variability among isolates of *A. solani* from tomato. *Indian Phytophathol*, *63*(2): 168-173.

Nikam, P. S., Suryawanshi, A. P., and Chavan, A. A. (2015). Pathogenic, cultural, morphological and molecular variability among eight isolates of *Alternaria solani*, causing early blight of tomato. *African journal of Biotechnology*, *14*(10), 871-877.

Rahmatzai, N., Zaitoun, A. A., Madkour, M. H., Ahmady, A., Hazim, Z., and Mousa, M. A. (2016). Research article morphological, pathogenic, cultural and physiological variability of the isolates of *Alternaria solani* causing early blight of tomato.

Simmons, E. G. (2007). *Alternaria*: An Identification Manual. CBS Biodiversity Series No. 6, Utrecht, the Netherlands, p. 775

Tamura, K. (1992). Estimation of the number of nucleotide substitutions when there are strong transition-transversion and G+ C-content biases. *Mol Biol Evol*, *9*(4), 678-687.

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F., and Higgins, D. G. (1997). The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic acids research*, *25*(24), 4876-4882.

Yunhui, T., Jinong, L., and Jingyou, X. (1994). A study on the biology and pathogenicity of Alternaria solani on tomato. *Journal of Jiangsu Agricultural College*, *15*(3), 29-31.

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