Int.J.Curr.Microbiol.App.Sci (2020) *9*(12): 2603-2609

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 9 Number 12 (2020)**
Journal homepage: http://www.ijcmas.com

Original Research Article https://doi.org/10.20546/ijcmas.2020.912.308

Characterization of *Alternaria* Species Causing Early Blight of Tomato

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Keywords

Tomato,
Early blight,
Alternaria solani,
Alternaria alternata

Article Info

Accepted:
18 November 2020
Available Online:
10 December 2020

A B S T R A C T

The present study aims at isolation, identification and characterization of different isolates of *Alternaria* spp from early blight infected seed samples collected at different regions of Tamilnadu. Four different isolates of *Alternaria* spp were obtained in pure culture which were designated as isolate PKM-CBE, PKM-DHR, PKM-DKL and PKM - KKR. Significant morphological variations in length and breadth of conidia, numbers of horizontal, vertical and oblique separations were observed in the test isolates. Isolates produce cotton mycelia growth with pigmentation varied from yellow, brown, and grey on potato dextrose agar medium. The PCR amplification of the fungal DNA using universal primers ITS1 and ITS4 and sequencing indicated that, isolates PKM-CBE, PKM-DHR, PKM-DKL were *Alternariasolani* and isolate PKM – KKR was *Alternaria alternata*. Pathogenicity test on tomato indicated that both *Alternaria* spp isolates were virulent.

Introduction

Tomato (*Solanum lycopersicum* Mill.) is one of the most popular vegetable crops grown globally. *Alternaria* is a cosmopolitan fungus and is the causal organism of leaf blight diseases in Cucurbitaceous and Solanaceous vegetables and accounted for 78 % yield loss at 72 % disease intensity (Datar and Mayee, 1981). The pathogen of the disease is reported as *Alternaria solani* (Alhussan, 2012; Derbalah *et al.*, 2011; Kumar *et al.*, 2008) and *Alternaria alternata* (Murugan *et al.*, 2014; Bhatt *et al.*, 2008). 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 diverse locations of

Tamilnadu were used to characterize *Alternaria* spp. based on cultural characteristics, pathogenic potentiality and ITS region.

Materials and Methods

Early blight infected tomato seed samples were collected from different regions of Tamilnadu and pure pathogen cultures were isolated on potato dextroseagar (PDA) and subsequently purified by single hyphal tip method. The cultures were transferred on PDA slants and incubated at 25 ± 2°C under alternate light/darkness (12 h each) for 72 h and were stored at 4°C for further use. Koch's postulates were tested for all the cultures.

Characterization based on Culture and Spore Morphology

Pure cultures of isolates were individually transferred onto PDA in petri dishes and incubated at25 ± 2°C. After 9 days, the cultures were observed for morphological characters *viz*., culture colour, growth pattern and spore morphological studies. The culture plates were incubated at 25 ± 2°C under alternate light/darkness (12 h each). After 9 days, for each culture, the spore morphologies were observed under microscope (Murugan *et al.*, 2014)

Molecular Characterization

DNA Extraction

Fungal isolates were grown on potato dextrose broth at25 ± 2°C for 10 days. DNA was extracted from the cultures. Mycelium of the cultures was taken out by filtration through Whatman No. 1 filter paper and washed thoroughly in distilled water and properly dried. 3g of mycelium was macerated along with liquid nitrogen with the help of mortar and pestle and transferred into a centrifuge tube and added with 15 mL of CTAB buffer (2 % (w/v) CTAB,1.4 M NaCl, 20 mM EDTA, 100 mM Tris–HCl, pH 8.0,1 % PVP, 1 % (v/v) 2-mercaptoethanol). The mixture was incubated at 65°C in a water bath for 30 min with intermittent shaking and the entire content was centrifuged at13,000 rpm for 10 min at 4°C. The supernatant was transferred into a fresh Oakridge tube and an equal volume of Phenol:Chloroform: Isoamylalcohol (25:24:1) was slowly added to it. Again, the content was centrifuged at 13,000 rpm for 10 min at 4°C. The supernatant was transferred into a fresh tube and added with 0.6 volume isopropanol and incubated overnight at -20°C. On the following day, it was again centrifuged at 13,000 rpm for10 min at 4°C temperature. The pellet was retained and supernatant was discarded. The pellet was briefly washed with 75 % ethanol and dried at room temperature. Finally the pellet was dissolved in 100 µL of TE buffer and kept at-20°C for further use.(Manicom *et al.*, 1987)

PCR

The fungal DNA was amplified using universal internal transcribe spacer region (ITS) primers (ITS1-TCCGTAGGTGAACC TGCGG and ITS4-TCCTCCGCTTATTGAT ATGCA) as described by (White *et al.*, 1990). The PCR reaction was carried out in a Thermocycler with 35 cycles of denaturation for 1 min at 94°C, primer annealing for 45 s at55°C and primer extension for 1 min 30 s at 72°C, with an initial denaturation at 94°C for 3 min and a final extension for15 min at 72°C. The reaction was carried out in a volume of25 µL containing 1 µL DNA template, 1.5 U *pfu* DNA polymerase,25 mM MgCl2, 2 mM dNTPs and 25 pmol of each primer. PCR products were electrophoresed (1 h at 80 volts) in0.8 % agarose gel in Tris–borate-EDTA buffer at pH 8. Gels were stained with ethidium bromide (10 µg/mL) and viewed in Gel documentation system.

Cloning of PCR product and sequencing

The PCR amplified product was excised from gel and purified by Gel extraction kit and cloned. Recombinant clones were sequenced with automated sequencer at Barcode Bio Sciences India Pvt. Ltd DNA Sequencing facility, Banglore, India.

Sequence analysis

The sequences obtained from the ITS region of all *Alternaria* isolates were subjected to NCBI (www.ncbi.nlm.nih.gov) BLAST search and the sequences showing highest scores were retrieved. The phylogenetic tree was generated using the neighbour joining method to estimate evolutionary distances.

Analysis of pathogenic variability

In order to test aggressiveness, the cultures were inoculated on tomato seedlings grown in pots filled with sterile potting mixture (Soil: Sand: well decomposed farm yard manure at ratio 2:1:1). For this study cultivarsPKM-1 seedlings were raised in pots. After25 days, seedlings were transplanted in 30 cm diameter pots.

Three seedlings were planted at equal distance in each pot. After 20 days of transplanting (45 days old), the plants were spray inoculated with spore suspension of fungal cultures. For preparation of spore suspension, the cultures were established on readymade V-8 agar medium containing asparagine as described in spore characterization study.

After 10 days, culture mat was harvested by applying10-15 ml of sterile water and scraping the mycelial mat with spores. To harvest the spores, the content was mixed well and filtered through three layers of sterile muslin cloth. The filtrate containing spores was adjusted to 10^5 spores/ml by diluting with sterile distilled water.

The spore suspension was spray inoculated on seedlings. After 5, 10 and 15 days of spray inoculation, the plants were observed for early blight development and scored for per cent disease index as described by Pandey *et al.*, 2003.

Results and Discussion

Cultural characterization

All the isolates proved Koch's postulates. Characterization of the isolates indicated that most of the cultures were grey or brown with regular growth pattern. Regarding conidial morphologies *viz*., conidial length, breadth and beak length. Among the isolates, conidial length was maximum in PKM - KKR isolate followed by PKM - DHRisolate whereas others were having significantly smaller size. Similarly, significant difference was observed in beak length (Table 1).

Most of the reports available in India indicated that the early blight of tomato is caused by *Alternaria solani* (Kumar *et al.*, 2008; Naik *et al.*, 2010) but the present study showed two species of *Alternaria* infecting tomato and hence to further confirm, molecular based identification of *Alternaria* isolates was undertaken.

PCR amplification and phylogenetic analysis

The early blight pathogen isolates collected from different geographical locations were amplified by PCR using universal primer pairs ITS1/ITS4. The process resulted in amplification of *580 bp in all infected tomato samples which corresponds to ITS rRNA of the fungal species. The PCR products were sequenced and nucleotide

sequences of ITS region of all isolates of *Alternaria*spp were compared with the representative ITS region of selected fungal species from the GeneBank database using BLAST (Table 2 and Figure 1).

The study clearly indicated that majority of *Alternaria* spp infecting tomato are *Alternaria solani* followed by *Alternaria alternata*. Similar identification of fungal pathogens through phylogenetic relationship was also successfully documented (Wang *et al.*, 2001; Pryor and Michailides, 2002).

Table.1 Cultural and morphological characteristics of *Alternaria* spp infecting tomato

Alternaria Isolate	Culture pigmentation	Growth pattern	Conidia				
			Length (µm)	Breadth (µm)	Beak length (µm)	Horizontal septa	Vertical septa
PKM-CBE	Yellowish grey	Regular	31.4	17.1	12.9	4.1	2.4
PKM-DHR	Grey	Regular	42.9	25.0	16.1	5.6	3.2
PKM-DKL	Dark grey	Regular	36.3	19.8	13.3	4.4	2.1
PKM - KKR	Brown	Regular	45.7	28.2	19.0	6.2	3.5
Mean			39.08	22.53	15.34	5.08	2.80
SEd			0.94	0.57	0.27	0.15	0.03
C D at 5%			2.17	1.32	0.63	0.34	0.07

Table.2 *Alternaria* isolates identified based on ITS sequencing and their NCBI accession numbers

c	Species	NCBI accession number
PKM-CBE	*Alternaria solani*	MN080230
PKM-DHR	*Alternaria solani*	MN080225
PKM-DKL	*Alternaria solani*	MN080229
PKM - KKR	*Alternaria alternata*	MN080226

Table.3 Disease scale description of early blight of tomato

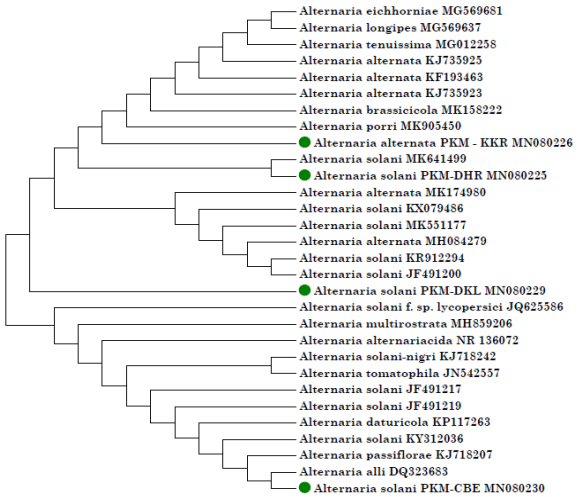
Scale	Leaf area infected (%)
0	Disease free
1	1-10 %
2	11-25 %
3	26-50 %
4	51-75 %
5	>76 %

Table.4 Aggressiveness of *Alternaria* isolates on tomato cultivar PKM 1

Isolate	Species	Percent Disease Incidence			Virulence category
		5 DAI	10 DAI	15 DAI	
PKM-CBE	*Alternaria solani*	16.9	38.3	78.1	Virulent
PKM-DHR	*Alternaria solani*	21.2	46.9	86.3	Virulent
PKM-DKL	*Alternaria solani*	17.1	37.8	75.9	Virulent
PKM - KKR	*Alternaria alternata*	19.9	43.4	80.5	Virulent
Mean		18.78	41.60	80.20	
SEd		0.29	0.67	1.14	
C D at 5%		0.66	1.55	2.61	

* DAI – Days after Inoculation

Fig.1 Phylogenetic tree showing relationships among the *Alternaria* spp based on their ITS sequences



Aggressiveness of *Alternaria solani* and *Alternaria alternata* on Tomato cultivar PKM 1

Though two species *Alternaria solani* and *Alternaria alternata* were observed in association of early blight of tomato, it is essential to study their potentiality to cause disease because in many instances these species exists as saprophytes or weak parasite or opportunistic pathogen (Guo *et al.,* 2004). Observations on virulence of different isolates of *Alternaria solani* and *Alternaria alternata* revealed that the species could produce similar kind of reaction on tomato cultivar PKM 1 (Table 3 and 4). However, the isolates belonging to same species manifested observable variation in pathogenicity. The study indicated that both *Alternaria solani* and *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) and Bhatt *et al.,* (2000) have reported the association of *Alternaria alternata* with early blight disease of tomato.

In conclusion the molecular and pathogenic characterization of *Alternaria* spp infecting tomato in Tamilnadu revealed the presence of

Alternaria solani and *Alternaria alternata* in virulent form and it emphasized that both *Alternaria* species needs to be considered for future early blight management or resistance breeding programmes.

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How to cite this article:

Ragupathi, K. P., P. R. Renganayaki, S. Sundareswaran, S. Mohan Kumar and Kamalakannan, A. 2020. Characterization of *Alternaria* Species Causing Early Blight of Tomato. *Int.J.Curr.Microbiol.App.Sci.* 9(12): 2603-2609.
doi: https://doi.org/10.20546/ijcmas.2020.912.308