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Morpho-molecular identification of *Alternaria alternata* associated with early blight of tomato (*Solanum lycopersicum* L.)

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Abstract
Early blight is one of the most damaging diseases in terms of both qualitatively and quantitatively in tomato producing areas worldwide. The disease was earlier reported to be incited by *Alternaria solani*, but several studies have shown that the disease is caused by several species of *Alternaria*. The present study was conducted to decode the species associated with early blight of tomato, samples showing blight appearance were collected from four districts of Jammu region and characterized through cultural and morphological characters. Based on cultural and morphological characteristics, the species was identified as *Alternaria alternata*, however, very little variation existed among the isolates. The species was further confirmed molecularly using universal internal transcribed spacer primer pairs. The pathogen showed 99% sequence similarity with *Alternaria alternata* and clustered in same clad in phylogenetic tree along with other *Alternaria alternata* isolates. The study clearly indicated the association of *Alternaria alternata*, in addition to *Alternaria solani* as the causal agent of early blight in tomato; the future prospects lies in collection of more number of isolates and using multigene approach for characterizing the species spectrum associated with early blight of tomato.

Keywords: Tomato, early blight, morphology, PCR, *Alternaria alternata*

Introduction
Tomato (*Solanum lycopersicum* Linnaeus), is one of the most commonly cultivated vegetable crop, grown in a wide range of climates from tropical to temperate regions in the world. It is the world's second most consumed vegetable after potato, and consumed in several ways: fresh, mixed in other food items or processed and canned as sauce, ketchup, juice, salsa, paste, soup and pickled [1] (Adhikari *et al.* 2017). It is the richest source of vitamin A and C and supplies a sufficient amount of the antioxidant lycopene pigment that helps to protect the body against cancer and heart disease [2]. Because of its wide use and nutritional values, there is a high demand for both fresh market and processed tomato varieties. However, it is susceptible to a number of diseases, but early blight caused by several *Alternaria* species is one of the important and economically significant, causing losses up to 78% [3]. Losses due to early blight in USA, Australia, Israel, UK, Brazil and India ranged from 35 to 80% [4, 5, 6]. The characteristic symptoms of the disease include dark brown to black concentric rings, which produces target board or bull's eye effect. Under favorable climatic conditions, the disease appears on stems, leaves, petiole, fruits, and twigs resulting in a premature fruit drop, twig drying and defoliation [7]. Initially, the symptoms of the disease appear on the leaflets as dark to brown leathery necrotic spots [8] which later becomes brightened and plants gradually defoliate [9, 10]. Several *Alternaria* spp. have been associated with early blight of tomato. The causal agent of early blight of tomato and other *Solanaceae* crops was initially considered to be *A. solani* Sorauer and has also been documented globally [11, 12, 13] as well as in India [14, 15, 16]. Akhtar *et al.,* (2004) [17] reported that *Alternaria alternata* is causing leaf blight of tomato in Pakistan. Three small spored *Alternaria* species such as *A. alternata* Keissler, *A. tenuissima* Nees & Nees: Fries, and *A. arborescens* (syn *A. alternata* f. sp. *lycopersici*) Simmons, have been repeatedly isolated from *Solanaceae* with symptoms resembling early blight at least at the initial stages of infection. Identification of early blight pathogen is generally based on conidial morphology, but for conidial production, the pathogen needs specific media, temperature, relative humidity and light.

Genetic identification is progressively used to identify the pathogens [18, 19] and internal transcribed spacer (ITS) rRNA has been successfully employed to identify the fungal pathogens at the species level [20, 21, 22]. In the present study, *Alternaria* blight infected tomato samples collected from diverse locations of Jammu province were used to characterize the association of *Alternaria* spp., based on cultural, morphological, pathogenic and molecular characteristics.

Material and Methods

Sample collection, isolation, purification and identification of the pathogen

Tomato leaves showing typical blight symptoms were collected from four tomato growing districts of Jammu province *viz.* Kathua, Doda, Udhampur and Jammu during cropping season of 2017-18. A total of twenty five blight infected samples were collected from each district and each isolate was coded as shown in table 1. The standard tissue isolation technique was followed for isolation of pathogen from fresh infected leaves [23]. Pure cultures were obtained using the hyphal tip technique [24] and a single spore culture technique [25]. The pure cultures obtained were maintained by repeated sub-culturing at an interval of 30 days for further studies.

Table 1: Cultural and morphological characteristics of 10 culture isolates collected from different places of Jammu

Isolate code	Collection site	Colony diameter (mm)	Colony color	Mycelial growth	Margin and zonation	Conidia width (µm)	Conidia length(µm)		Beak length (µm)	Number of septa		
							With beak	Without beak		Vertical	Horizontal	Oblique
AS1	Chatha	73.21	Juniper Green	Regular smooth	Circular and Concentric	9.53	42.47	37.01	4.61	6	1	0
AS2	R.S. Pura	72.74	Juniper Green	Regular smooth	Circular and Concentric	9.55	41.01	37.23	4.29	6	0	0
AS3	Mishriwala	72.54	Dark Green	Regular smooth	Diffused with Zonation	9.00	42.77	38.71	3.76	5	0	1
AS4	Akhnoor	70.11	Juniper Green	Regular smooth	Circular and Concentric	9.02	42.34	37.55	4.01	6	1	0
AS5	Marh	72.61	Juniper Green	Regular smooth	Circular and Concentric	9.23	42.65	38.00	4.23	6	1	0
AS6	Bagar	71.63	Juniper Green	Regular smooth	Circular and Concentric	9.25	42.72	38.21	3.90	6	0	0
AS7	Asar	72.25	Dark Green	Regular smooth	Circular and Concentric	9.32	41.24	38.43	4.62	6	0	0
AS8	Barmora	71.56	Juniper Green	Regular smooth	Circular and Concentric	9.04	42.05	37.03	4.75	6	0	0
AS9	Jangicheck	73.24	Juniper Green	Regular smooth	Circular and Concentric	9.64	42.63	37.55	4.70	5	0	1
AS10	Chennani	72.33	Juniper Green	Regular smooth	Circular and Concentric	9.43	42.26	38.65	3.44	6	1	0

Morphological characterization

Cultural characters were recorded from 7 day old inoculated isolates. The variability of representative isolates of *A. solani* was studied on various parameters of cultural characters such as colony color, mycelial growth, margin, zonation and colony diameter by direct observation of culture grown Petri plates. Sporulation was recorded on PDA tested media using 7 days old culture under the microscope. The isolates were tested for their cultural and morphological variations on PDA. Spores of fungus were taken from the pure culture and mounted on the clear glass slide thoroughly mixed with lacto phenol to obtain a uniform spread over the slide. The spores and hyphae of the fungus were observed under a compound microscope and the fungus was identified following the cultural and morphological characteristics criteria as described by [26, 27].

Molecular characterization

Based on the morphological and cultural study, three representative cultures were selected for their molecular validation.

DNA isolation, PCR amplification and gel electrophoresis

The genomic DNA of the representative cultures was extracted by using Quick DNA^{TM} fungal Miniprep isolation kit (ZYMO Research). The PCR was carried out in a final volume of 40 µl, containing ITS-1 (5'GGAAGTAAAAGTCGTAACAAGG3') forward primer (1µl), ITS-4 (5'TCCTCCGCTTATTGATGATATGC3') reverse primer (1µl), template DNA (3µl), Taq master mix (20 µl) (Roche Applied Science, Penzberg, Germany) and water (15 µl). The reactions were carried out in a Peltier thermo cycler (PTC) for 35 cycles, with initial denaturation of 94 °C for 5 minutes, 94 °C for 1 minute, 53 °C for 45 seconds (annealing temperature), 72 °C for 1 min (extension) and 72 °C for 10 minutes (final extension) [28]. The amplicon were subjected to 1.5% gel electrophoresis in order to visualize the bands. The 100 base pairs (bp) ladder was used as a size standard. DNA was visualized under UV trans-illuminator. The fluorescing bands of PCR products were photographed using gel documentation system.

Sequencing, BLAST and phylogenetic analysis

Amplicons were sequenced at Xcelris Genome Labs (Gujarat) and PCR products (sequences) were assembled using the DNA Baser V.4 program to produce complete contigs. These were further aligned using the Clustal W method (Bio-Edit). A search of homologous sequences was performed by BLAST analysis at NCBI (http://ncbi.nlm.nih.gov/BLAST). The MEGA7 (Molecular Evolutionary Genomics Analysis Version 7) (22) constructed dendograms from three isolates from the current study and reference strain sequences from

Gen Bank. The sequences were submitted to Gen Bank.

Pathogenicity test

In order to confirm the pathogenicity of the isolates, the pathogenicity test was carried out by atomizing the conidial suspension 3x10^6 spores' ml^{-1} @ 8-10mL/plant. The spore suspension was, prepared from 10-days old culture and 30 days old seedlings of susceptible variety Pusa ruby was inoculated. The symptoms expressed were studied and re-isolation of fungus was done to prove the Koch's postulates.

Results and Discussion

Disease incidence and Symptomatology

During the course of survey, the mean incidence and intensity of leaf blight recorded from all districts was high, varied from 25 to 30% and 12 to 17%. The symptoms observed were brown necrotic lesions and were visible on the older leaves, advancing upwards as the plants become older (Figure 1). The lesions were oval to angular in shape with 1-2 mm in diameter and concentric rings surrounded by a yellow chlorotic halo were formed. The severe phase of the disease leads to premature defoliation and drying of the plant. Walker (1952) [29] also observed the similar symptoms described the spots as oval or angular in shape 0.3 or 0.4 cm diameter in size with usually narrow chlorotic zone around and confirmed the disease as early blight. Similarly, several workers also reported that the symptoms of early blight as small, dark, necrotic brown color lesions that usually appear on the older leaves and spread upwards as the plant ages, which corroborated with our results [30, 31, 32].

Fig 1: Symptoms of early blight on leaves of tomato observed during field survey

Cultural characteristics

Fig 2: Colonies having a circular margin and whitish center exhibiting green color

Total 10 cultures were purified and studied for various cultural characters *viz.*, colony diameter, colony color, mycelial growth, margin and zonation. The results revealed least variations among tested isolates. The maximum colony diameter (73.21 mm) was recorded in isolate AS1 (Figure 2), while minimum in isolate AS4 (70.11 mm). In case of colony color, isolates exhibit juniper green to dark green color. No variation was observed in mycelial growth and margin pattern as all the isolates have a circular margin. In case of zonation, the isolates were found to exhibit a concentric zonation pattern. The above parameters recorded were in agreement with the previous findings on cultural variability of *Alternaria alternata* (Hubballi *et al.,* 2010) [33]. The cultural characters of all the 10 isolated cultures are shown in table 1.

Morphological characteristics

The morphological characters of all 10 isolates exhibited less variation with respect to conidia septation (horizontal/vertical/oblique), beak length, presence or absence of beak, length and width among the fungal isolates. The conidia were ovoid or ellipsoidal and found in long chains with short beak (Figure 3). The maximum length of the conidia along with the beaks as recorded in isolate AS1 (42.47 µm) with six horizontal and one vertical septa and the minimum length of the conidia with beak was observed in the isolate AS23 (41.01 µm) along with six horizontal septa. In case of beak length, the maximum beak length was observed in isolate AS9 (4.70 µm) and the minimum beak length was recorded in isolate AS10 (3.44 µm). Conidial width also varies, as maximum conidial width was found in isolate AS1 (9.53 µm) and a minimum width in isolate AS4 (9.02 µm). The morphological characters of all isolates are shown in table 1. The fungus *Alternaria alternata* was identified based on morphological characters [26]. These results are also in conformity with the separate findings of various workers [34.17], where morphological characters of the fungus on PDA included effuse, circular & olivaceous green colonies; branched, flexuous conidiophores arising from scars; conidia in long chains, obpyriform with a short cylindrical beak, mild golden brown, smooth with 5-6 transverse and usually many longitudinal septa, conidia measuring about 40 µm in length and about 15 µm thick in the broadest part.

Fig 3: Conidia ovoid or ellipsoidal with short beak observed under microscope at 40 x resolution

Molecular identification

The representative to confirm the species through molecular approach, isolates namely AS22, AS23 and AS25 were amplified by PCR using universal primer pairs ITS1 / ITS4

which resulted in amplification of 560 bp (Figure 4) in all the three fungal isolates. The PCR products were sequenced and nucleotide sequences of ITS region after BLAST analysis showed 99% sequence homology with already submitted *Alternaria alternata* sequences in Gen Bank. The sequences of all the three isolates were submitted to Gen Bank with accession Numbers MN093344, MN097247, MN093376. The phylogenetic tree (Figure 5) was developed using MEGA 7.0 software and all three isolates were clustered along with other *Alternaria alternata* isolates.

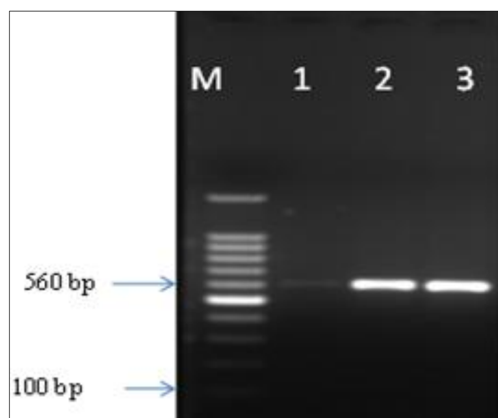

Fig 4: PCR product (560 bp) amplified from ITS region in all 3 *Alternaria alternata* isolates L-1kb ladder, 1-3: *Alternata alternata* isolates from various locations

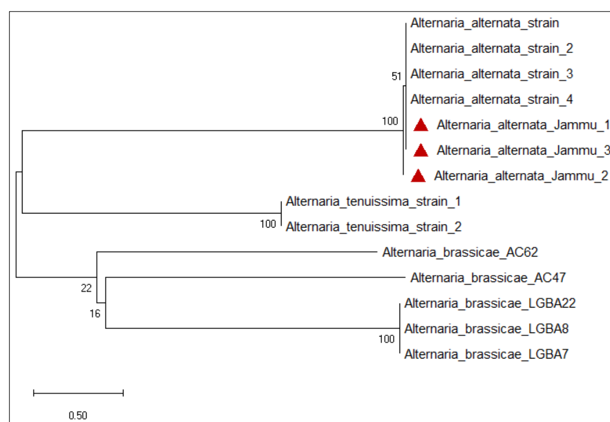

Fig 5: Phylogenetic relationship of *Alternaria alternata* using the internal transcribed spacer (ITS) region gene nucleotide sequence alignment with bootstrap values at nodes using Neighbor joining method

Pathogenic test

Pathogenicity test was carried out by atomizing the conidial suspension $3x10^6$ spores per ml @ 8-10 mL/plant, prepared from 10-day old culture on foliage of 30 days old seedlings of susceptible variety Pusa ruby. After one week of inoculation, the symptoms on the leaves appeared as dark brown angular or oval necrotic spots having concentric rings on foliage surrounded by chlorotic yellow halo zone. Re-isolation was made from the test plants to prove the Koch's postulates and fungus from artificially inoculated plants revealed cultural and morphological similarity to original ones. No symptoms were observed on un-inoculated tomato plants.

Conclusion

Early blight caused by various species of *Alternaria* is one of the economically significant diseases, causing huge losses. Earlier, only *Alternaria solani* was associated with early blight of tomato, but afterwards several different *Alternaria* spp. have been associated with early blight of tomato (Simmons 2000) [26]. During current study *Alternaria alternata* was identified to be associated with early blight disease in tomato, based on morphological, molecular characterization. But the need of the hour is to apply multilocus gene with more isolates to further resolve the identity of *Alternaria spp* associated with early blight disease of tomato. Further, the present study opens new vistas in better management of early blight especially in endemic areas and identification of resistant donors against this disease against different species of *Alternaria*.

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References

1. Adhikari P, Oh Y, Panthee DR. Current status of early blight resistance in tomato: An Update. Int J Mol Sci. 2017;18:1-20.
2. Bohm F, Edge R, Truscott G. Interactions of dietary carotenoids with activated (singlet) oxygen and free radicals: Potential effects for human health. Mol. Nutr. Food Res. 2012;56:205-216.
3. Chaerani R, Voorrips RE. Tomato early blight (*Alternaria solani*): the pathogen, genetics, and breeding for resistance (review). Journal of General Plant Pathology. 2006;72:335-347.
4. Basu PK. Measuring early blight, its progress and influence on fruit losses in nine tomato cultivars. Canadian Plant Disease Survey. 1974;54:45-51.
5. Datar VV, Mayee CD. Assessment of loss in tomato yield due to early blight. Indian Phytopathol. 1981;34:191-195.
6. Grigolli JFJ, Kubota MM, Alves DP, Rodrigues GB, Cardoso CR, Henriques da Silva DJ, *et al.* Characterization of tomato accessions for resistance to early blight. Crop Breeding and Applied Biotechnology. 2011;11;174-180.
7. Mathur K. and Shekhawat K.S. Chemical control of early blight in Kharif sown tomato. Indian Journal of Mycology Plant Pathology. 1986;16:235-238.
8. Locke S.B. Resistance to early blight and septoria leaf spot in the genus Lycopersicon. Phytopathology. 1949; 89:829-836.
9. Thomas H.R. Effect of Nitrogen, Phosphorous and potassium on susceptibility of tomatoes to *Alternaria solani*. Journal of Agriculture research. 1948;76:289-306.
10. Barksdale T.H. and Stoner A.K. A study of inheritance of tomato early blight resistance. Plant Dis Rep. 1977;61:63 -65.
11. Gomes SMDTP, Carneiro EDB, Romano EP, Teixeira MZ, da Costa ME, Vasconcelos JCG. Effect of bio therapy of *Alternaria solani* on the early blight of tomato-plant and the in vitro development of the fungus. Int J High Dilution Res. 2010;9:147-155.
12. Derbalah AS, El-Mahrouk MS, El-Sayed AB. Efficacy and safety of some plant extracts against tomato early blight disease caused by *Alternaria solani*. Plant Pathol J

2011;10(3):115-121.
13. Alhussaen KM. Morphological and physiological characterization of *Alternaria solani* isolated from Tomato in Jordan Valley. Research Journal of Biology Science. 2012;7:316-319.
14. Varma KP, Singh S, Gandhi SK. Variability among *Alternaria solani* isolates causing early blight of tomato. Indian Phytopathol. 2007;60:180-186.
15. Kumar V, Haldar S, Pandey KK, Singh RP, Singh AK, Singh PC. Cultural, morphological, pathogenic and molecular variability amongst tomato isolates of *Alternaria solani* in India. World J Microbiol Bio technol, 2008;24:1003-1009.
16. Radhajeyalakshmi R, Velazhahan R, Samiyappan R, Doraiswamy S. Systemic induction of pathogenesis related proteins (PRs) in *Alternaria solani* elicitor sensitized tomato cells as resistance response. Sci Res Essay. 2009;4:685-689.
17. Akhtar KP, Saleem MY, Asghar M, Haq MA. New report of *Alternaria alternata* causing leaf blight of tomato in Pakistan. Plant Pathology: 2004;53:816.
18. Vunch R, Rosner A, Stein A. The use of the polymerase chain reaction (PCR) for detection of bean yellow mosaic virus in gladiolus. Ann Appl Biol. 1999;117:561-569.
19. Bridge PD, Singh T, Arora DK. The application of molecular markers in the epidemiology of plant pathogenic fungi. In: Arora DK, Bridge PD, Bhatnagar D (eds) Fungal biotechnology in agricultural, food, and environmental applications. Marcel Dekker Inc, New York; c2004. p. 475.
20. Abd-Esalem KA. Non-gel based techniques for plant pathogen genotyping. Acta Microbiol Pol. 2003;52:329-341.
21. Abd-Esalem KA, Asran-Amel A, Schneider F, Migheli Q, Verreet JA. Molecular detection of *Fusarium oxysporum*. f. sp. *vasinfectum* in cotton roots by PCR and real-time PCR assay. J Plant Dis Prot. 2006;113:14-19.
22. Bowmann KD, Albrecht U, Graham JH, Bright DB. Detection of *Phytophthora nicotianae* and *P. palmivora* in citrus roots using PCR-RFLP in comparison with other methods. Eur J Plant Pathol. 2007;119:143-158.
23. Naik MK, Prasad Y, Bhat KV, Rani D. Morphological, physiological, pathogenic and molecular variability among isolates of *Alternaria solani* from tomato. Indian Phytopathol. 2010;63(2):168-173.
24. Pathak, V.N. Essentials of Plant Pathology, Prakesh Publishers, Jaipur, India; c1972. p. 448
25. Johnston A Booth C. Plant pathologist's pocketbook. 2nd ed. Kew: Commonwealth Agricultural Bureaux, The Commonwealth Mycological Institute; Ellis M.B. Dematiaceous Hyphomycetes. Commonwealth Mycological Institute, Kew, Surrey, UK; c1971. p. 608.
26. Simmons E.G. *Alternaria* themes and variations (244-286) species on Solanaceae. Mycotaxon. 2000;75:1-115.
27. Gherbawy H, Hussein MA, Runge F, Spring O. Molecular characterization of *Alternaria alternata* population isolated from Upper Egyptian tomato fruits. Journal of Phytopathology. 2017;166:709-7214402.
28. Walker JC. Diseases of Vegetable Crops, 1st ed. MacGraw-Hill Book Company, Inc. New York. Wheeler, B.E.J. 1969. An Introduction of Plant Diseases, pp.301. John Willey and Sons Ltd. London; c1952. p.471-474.
29. Sherf AF, MacNab AA. Vegetable diseases and their control, John Wiley and Sons, New York; c1986 p. 634-640.
30. Singh RS. Diseases of Vegetable Crops, pp.419. Oxford and IBH Pub. Co. Pvt. Ltd., New Delhi, Bombay, Calcutta; c1987.
31. Peralta IE, Knapp S, Spooner DM. New species of wild tomatoes (*Solanum* section *Lycopersicon*: Solanaceae) from Northern Peru. Sys Bot. 2005;30:424-434.
32. Hubballi M, Nakkeeran S, Raguchander T, Anand T, Samiyappan R. Effect of environmental conditions on growth of *Alternaria alternata* causing leaf blight of onion. World Journal of Agricultural Sciences. 2010;6: 171-177.
33. Bhatt JC, Gahlain A, Pant SK. Record of *Alternaria alternata* on tomato, capsicum and spinach in Kumaon hills. Indian Phytopathol. 2000;53:495-496.