

Full Length Research Paper

Pathogenic, cultural, morphological and molecular variability among eight isolates of *Alternaria solani*, causing early blight of tomato

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Among the fungal diseases infecting tomato crops, early blight caused by *Alternaria solani* (Ellis and Martin) Jones and Grout is one of the most catastrophic disease causing accountable losses. Further, all of the tomato cultivars presently under cultivation have succumb more or less to early blight disease. Therefore, the present studies were undertaken for the pathogenic, cultural, morphological and molecular variability among the isolates of *A. solani*. The results reveal all of the eight isolates of *A. solani* as pathogenic to tomato (Cv. Pusa Ruby) and showed variability amongst them. The test isolates could grow better on the basic culture medium potato dextrose agar; however, highest mycelial growth was recorded on the isolate AsLt (88.50 mm), followed by AsBd (82.36 mm) and AsHI (78.40 mm), with excellent sporulation. All of the eight test isolates exhibited a wide range of variability in respect of their mycelial and conidial dimensions and septation. RAPD-PCR analysis of the four most virulent *A. solani* isolates, using 13 OPA primers revealed that the isolates AsBd (Beed) and AsLt (Latur) were closely related with 85% genetic similarity whereas, the isolates AsHI (Hingoli) and AsJI (Jalna) were closely related with 50% genetic similarity, but distinct from that of AsLt and AsBd isolates.

Key words: Tomato, *Alternaria solani*, isolates, pathogenic, molecular variability, virulent, primers.

INTRODUCTION

Tomato (*Solanum lycopersicon*) is one of the most popular vegetable crops grown throughout the world. India is one of the leading countries in tomato production with an area of 8.65 lakh ha and productivity of 19.5 metric tonnes/ha (Anonymous, 2011). Maharashtra, Bihar, Uttar Pradesh, Karnataka and West Bengal are the major tomato growing states in India. In Maharashtra it occupies an area of 5.20 thousand ha with the

productivity of 14.2 metric tonnes/ha (Anonymous, 2011). Diseases are the major constraints in tomato cultivation causing qualitative as well as quantitative losses. Among the fungal diseases infecting tomato crop, early blight caused by *Alternaria solani* (Ellis and Martin) Jones and Grout, is one of the most catastrophic disease. The disease induces the symptoms such as dark brown to black spots with concentric rings giving a target board

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effect. The disease early blight have been reported to inflict the yield losses to the tune of 48 to 80% (Datar and Mayee, 1984; Mathur and Shekhawat, 1986; Pandey and Pandey, 2002). Under natural epiphytotics, the pathogen (*A. solani*) have been found to express a wide range of variability in disease symptoms expression depending upon the tomato cultivars, environmental conditions etc. Pathogenic, cultural, morphological and molecular variability in *A. solani* was also documented earlier by many workers (Kaul and Saxena, 1988; Tong et al., 1994; Weir et al., 1998; Babu et al., 2000; Ahmad, 2002; Naik et al., 2010). The variability of *A. solani* isolates studied under greenhouse conditions of several isolates infecting 14 tomato genotypes has been reported earlier by Castro et al. (2000).

Therefore, the present investigations were attempted to explore the pathogenic, cultural, morphological and molecular variability among the eight isolates of *A. solani*, isolated from the tomato crop affected with early blight disease which were collected during survey in Marathwada region of the state of Maharashtra, India.

MATERIALS AND METHODS

Collection, isolation and maintenance of *A. solani* isolates

A roving survey of the tomato crop fields was undertaken during *Kharif* and *Rabi* seasons of 2010 to 2011. Tomato plant samples (leaves, fruits and branches) showing typical symptoms of early blight disease were collected from the eight districts viz., Parbhani, Nanded, Hingoli, Latur, Osmanabad, Beed, Aurangabad and Jalna in Marathwada region of the state of Maharashtra. These diseased samples were brought to the laboratory washed thoroughly under running tap water, blot dried, cut into small bits (2 mm), surface sterilized (0.1% HgCl₂), washed in three sequential changes of sterile water, blot dried, aseptically subjected to tissue isolation, using autoclaved and cooled Potato dextrose agar (PDA) in sterile glass Petri plates (90 mm dia.) and incubated at 27±2°C. After a week of incubation, the culture growth was aseptically transferred, applying hyphal tip method onto fresh PDA plates and incubated at 27±2°C temperature. Further, sub-cultured and the pure cultures of each isolate thus obtained were maintained separately on PDA slants in glass test tubes and stored in refrigerator for further studies.

Pathogenicity test and pathogenic variability

The pathogenicity tests for *A. solani* eight isolates were attempted under screenhouse, applying Koch's postulates. For the purpose, 30 days old potted seedlings of susceptible tomato Cv. Pusa Ruby were spray inoculated with the spore suspension (5 x 10⁶ spores / ml) of the test pathogen incubated in screen house and observed for the development of early blight. Disease incidence was rated as suggested by Pandey and Pandey (2002); symptoms expressed were studied and reisolated from the infected stem. The pathogenicity test as above was repeated twice to confirm results. Pathogenic variability of *A. solani* eight isolates was studied by applying detached leaf technique. The spore suspension (5 x 10⁶ spores / ml) of each test isolate was separately prepared from 5 days old pure culture of the representative isolates. Healthy growing five tomato leaves of tomato susceptible Cv. Pusa Ruby per moist chamber per isolate were inoculated separately with the

spore suspension (5 x 10⁶ spores / ml) and incubated at room temperature. Observations on incubation period, number of spots, frequency, size of lesions and typical symptoms induced etc. were recorded and based on these characteristics, the test isolates were categorized as highly virulent, moderately virulent and a mildly virulent.

Cultural variability

All of the eight test isolates of *A. solani* were isolated aseptically on the PDA plates, incubated at 27±2°C for a week and their cultural characteristics were observed viz., colony diameter, colony colour / pigmentation, mycelial growth etc., and sporulation was recorded after 15 days of incubation, by observing under research microscope (Make : Labomed, 2000).

Morphological variability

Morphological characteristics viz., mycelial width, size of conidia, length of beak and number of transverse and longitudinal septa of all the eight isolates of *A. solani* were measured using ocular micrometer (Calibrated using stage micrometer) under 400 x magnification of research microscope (Make : Labomed, 2000).

Molecular variability

Based on cultural, morphological and pathogenic studies of the eight isolates of *A. solani*, only four highly virulent isolates (AsLt, AsBd, AsJl and AsHl) were selected for studying their molecular variability applying the following procedures and protocols. The genomic DNA of the four test isolates of *A. solani* was extracted using the protocol developed and standardized by Chakrabarty (2003).

PCR amplification

PCR was carried out in a final volume of 25 µl containing 0.1 to 0.5 µm of oligonucleotide primer (2.0 µl), 25 mM of each of the deoxynucleotide triphosphates (0.2 µl), 50 mM MgCl₂ (1.5 µl), 0.3 µl of Tag DNA polymerase, 10 x PCR buffer (2.5 µl) and template DNA (100 ng / 0.5 µl). The reaction mixture was overlaid in PCR tubes except template DNA. Genomic DNA of each isolate was added to individual tube containing the water mix. Amplification was carried out in a thermocycler with initial denaturation at 94°C for 4 min, followed by 40 cycles of denaturation at 94°C for 1 min, primer annealing at 35°C for 1 min and primer elongation at 72°C for 2 min, followed by an extended elongation at 72°C for 10 min. In order to identify the most suitable RAPD primers for the study of molecular variations among the isolates, 13 random primers from OPA (Operon Technologies USA) series that is, OPA 1-13 were used for analyses of all the isolates. The amplified PCR products were separated on 1.2% agarose gel and visualized under illumination by staining with ethidium bromide and photographed by using Gel Doc. All amplifications were repeated at least twice and only reproducible bands were considered for analysis. The data was analyzed using the software NTSYS- pc to Plot Dendrogram.

RESULTS AND DISCUSSION

Pathogenicity test and pathogenic variability

Results of the pathogenicity tests for *A. solani* eight

Table 1. Cultural variability among the isolates of *A. solani*, causing early blight in tomato.

Isolates / districts	Colony diameter (mm)	Cultural characteristics	Colony colour/pigmentation	Sporulation
AsPb (Parbhani)	76.25	Circular, smooth, without zonation	Black	+++
AsNd (Nanded)	65.80	Circular, smooth, with concentric zonation	Brownish black	++
AsHI (Hingoli)	78.40	Irregular, smooth, with concentric zonation	Dark grayish	++++
AsLt (Latur)	88.50	Irregular, smooth, with concentric zonation	Brownish black	++++
AsOb (Osmanabad)	75.60	Irregular, smooth, without zonation	Olivaceous black	++
AsBd (Beed)	82.35	Circular, smooth with concentric zonation	Pinkish red	++++
AsAb (Aurangabad)	70.42	Irregular, rough, without zonation	Light gray	++
AsJI (Jalna)	78.20	Circular smooth, with concentric zonation	Creamish white	++++

Sporulation: +++++, excellent; +++, good; ++, fair; +, poor. Cultural characteristics, colony colour and sporulation were also varied for the test isolates. Irregular smooth colonies, with concentric zonation, brownish black (AsLt isolate) and dark grayish (AsHI isolate), without zonation olivaceous black (AsOb isolate) and light grey (AsAb isolate) colonies were produced whereas, circular, smooth colonies with concentric zonation brownish black (AsNd isolate), Pinkish red (AsBd isolate) and creamish white (AsPb isolate) colonies were produced by the rest of the four isolates. The sporulation induced by all test isolates varied from poor (+) to excellent (++++). However, the isolates viz., AsLt, AsHI, AsJI and AsBd showed excellent (++++) sporulation whereas, it was good (+++) in AsPb and fair (++) in isolates viz., AsNd, AsOb and AsAb.

isolates conducted in screen house using susceptible tomato Cv. Pusa Ruby revealed that after a week of incubation period typical symptoms of early blight on the foliage similar to these observed on tomato foliage naturally affected with *A. solani* under field conditions were produced. Based on the observation and recorded data, four isolates (AsLt, AsBd, AsHI and AsJI) were found to be highly virulent due to short incubation period (4 to 5 days), maximum number of lesion size (6 to 8 / mm²) and maximum average lesion size (12 to 15 / mm²). Other isolates were rated as moderately virulent and a mildly virulent because these isolates were unable to cause symptoms of the disease in greenhouse experiments. Therefore, only four most virulent isolates (AsLt, AsBd, AsHI and AsJI) of *A. solani* were subjected for molecular variability study. The variation in pathogenicity of *A. solani* isolates was also established by Tong et al. (1994). The results are also in accordance with those of Castro et al. (2000) who demonstrated the variability of *A. solani* isolates on 14 tomato genotypes and Verma et al. (2007) who reported that the test isolates differed in the virulence pattern on ten tomato genotypes under screen house conditions.

Cultural variability

The results (Table 1) reveal that all the eight isolates of *A. solani* exhibited a great variability in respect of mycelial growth, colony color, colony diameter, colony zonation and sporulation. Maximum mycelial growth was recorded by AsLt (88.50 mm), isolate, followed by AsBd (82.35 mm), AsHI (78.40 mm), AsJI (78.20 mm), AsPb (76.25 mm), AsOb (75.60 mm), AsAb (70.42 mm) and AsNd (65.80 mm). Cultural characteristics, colony colour and sporulation were also varied for the test isolates. Irregular smooth colonies, with concentric zonation,

brownish black (AsLt isolate) and dark grayish (AsHI isolate), without zonation olivaceous black (AsOb isolate) and light grey (AsAb isolate) colonies were produced whereas, circular, smooth colonies with concentric zonation brownish black (AsNd isolate), Pinkish red (AsBd isolate) and creamish white (AsPb isolate) colonies were produced by the rest of the four isolates. The sporulation induced by all test isolates varied from poor (+) to excellent (++++). However, the isolates viz., AsLt, AsHI, AsJI and AsBd showed excellent (++++) sporulation whereas, it was good (+++) in AsPb and fair (++) in isolates viz., AsNd, AsOb and AsAb. These results on cultural variability of *A. solani* isolates observed in the present study are similar to the findings of several earlier workers. Kaul and Saxcena (1988), based on cultural variability identified four isolates of *A. solani*. Similar cultural variability's among the isolated *A. solani* grown on PDA medium were reported earlier by several workers (Tong et al., 1994; Babu et al., 2000; Kumar et al., 2008; Naik et al., 2010).

Morphological variability

Studies on morphological characteristics of the eight test isolates of *A. solani* exhibited variations with respect to mycelial width, conidial size, beak length and septation (Table 2). Conidiophores were solitary or in small groups, straight or flexous, brown to olivaceous brown. The conidia were solitary, straight or slightly flexous, muriform and ellipsoidal tapering to a beak and pale or olivaceous brown. The mycelial width was found to be highest in AsJI (6.42 µm) isolate, followed by AsBd (6.20 µm), AsHI (4.50 µm) and AsLt (4.26 µm) isolates. Whereas, it was comparatively medium in the isolates viz., AsNd (3.73 µm), AsPb (3.64 µm) and AsAb (3.50 µm) while, least in AsOb (2.54 µm) isolate. The average conidial size (L x B)

Table 2. Morphological variability among the isolates of *A. solani*, causing early blight in tomato.

Isolate (district)	Mycelial width (μm)	Average size of conidia (μm)		Beak length (μm)	Number of septa	
		Length	Breadth		Horizontal	Transverse
AsPb (Parbhani)	3.64	18.30	9.50	8.48	3-8	0-2
AsNd (Nanded)	3.73	22.20	9.91	10.34	4-7	1-2
AsHl (Hingoli)	4.50	28.35	12.90	12.21	4-8	1-3
AsLt (Latur)	4.26	37.57	13.21	12.81	5-9	1-3
AsOb (Osmanabad)	2.54	13.25	7.68	8.35	1-2	0-1
AsBd (Beed)	6.20	42.18	15.18	13.10	5-12	1-4
AsAb (Aurangabad)	3.50	18.28	8.82	9.10	3-5	0-2
AsJl (Jalna)	6.42	25.83	11.90	11.61	4-8	1-3

and their beak length were highest in the isolate AsBd (42.18 x 15.18 and 13.10 μm , respectively), followed by the isolated *viz.*, AsLt (37.57 x 13.21 and 12.81 μm), AsHl (28.35 x 12.90 and 12.21 μm) and AsJl (25.83 x 11.90 and 11.61 μm) of conidial size and conidial beak length, respectively. Whereas, in rest of the three isolates AsAb, AsPb and AsNd, the conidial size and beak length were medium in the range of 18.28 x 8.82 to 22.20 x 9.91 μm and 9.10 to 10.34 μm , respectively, while, the isolate AsOb showed small sized conidia (13.25 x 7.68 μm) with short beak length (8.35 μm). The conidial septation (No. of horizontal and vertical septa) was also found to be varied among the test isolates. However, the septation was maximum in the isolate AsBd (5 to 12 and 1 to 4), followed by the isolates *viz.*, AsLt (5 to 9 and 1 to 3), AsHl and AsJl (each 4 to 8 and 1 to 3), AsNd (4 to 7 and 1 to 2) and AsPb (3 to 8 and 0 to 2) and AsAb (3 to 5 and 0 to 2) with number of horizontal and vertical septa, respectively, while it was merge in the isolate AsOb.

These conidial features of *A. solani* test isolates observed in the study are in accordance with the *A. solani* spores characteristics originally described by Ellis and Ellis (1985) whereas, Ahmad (2002) studied the variations in conidial morphology of *A. solani*, causing tomato early blight disease and reported comparatively large sized (175 x 12.5 μm) conidia and their larger beak length (47 to 65 μm), which are contradictory to the conidial measurements found in present study. Verma et al. (2007), Kumar et al. (2008) and Naik et al. (2010) also reported similar variations in the conidial morphology of the isolates of *A. solani*. These variations may be due to different kind of *A. solani* isolates that prevailed there, season and tomato cultivars grown under the environmental conditions prevalent there.

Molecular variability

Based on amount of mycelial growth, pathogenic, cultural and morphological variability, only four most virulent *A. solani* isolates *viz.*, AsLt, AsBd, AsJl and AsHl were

subjected to molecular variability study, using RAPD-PCR analysis and 13 random primers of OPA series (OPA 1 to 13). The RAPD analysis (Figure 1) revealed genetic variability in all four test isolates of *A. solani*. Of the 13 primers tested, primers *viz.*, OPA 4, OPA 7, OPA 9, OPA 11, OPA 12 and OPA 13 revealed amplifications. The primer OPA 4 and OPA 7 also generated multiple amplicons. Based on the efficiency of the individual primers to amplify polymorphic DNA fragment, six primers: OPA 4, OPA 7, OPA 9, OPA 11, OPA 12 and OPA 13 were selected for fingerprinting of all these species and provide representative profiles of isolates. The dendrogram constructed based on similarity coefficients grouped the isolates into two different groups at a similarity coefficient of 0.50 (Figure 2). Group I occupied by two isolates AsBd and AsLt and group II occupied by rest of two isolates AsHl and AsJl. The AsBd and AsLt were found more similar (85% similarity coefficient) to each other whereas, the isolates AsHl and AsJl were found closely related (50% similarity coefficient) but distinct from AsLt and AsBd. Thus, the present study based on RAPD-PCR analysis revealed that there may be chances of prevalence of the genetically variable populations amongst pathogen *A. solani*, of tomato crop grown in Marathwada region of Maharashtra State. However, isolates was found to be lower than the previous reports which may be attributed to less number of isolates used in this study.

Genetic variability in *A. solani*, causing early blight disease in tomato / potato crop grown in various regions was reported earlier by various workers (Weir et al., 1998; Morris et al., 2000; Wang and Zhang, 2003). These findings of the present study are in consonance with the earlier study of Verma et al. (2007), Kumar et al. (2008) and Naik et al. (2010). Naik et al. (2010) investigated genetic variations among four isolates of *A. solani* and indicated maximum similarity (73.78%) in isolates of Northern Karnataka region (ASR₅, ASD₄ and ASG₃) and isolate ASB2 from Southern Karnataka region shared only 45% genetic similarity indicating distinct polymorphism. Thus, from the foregoing results and discussion,

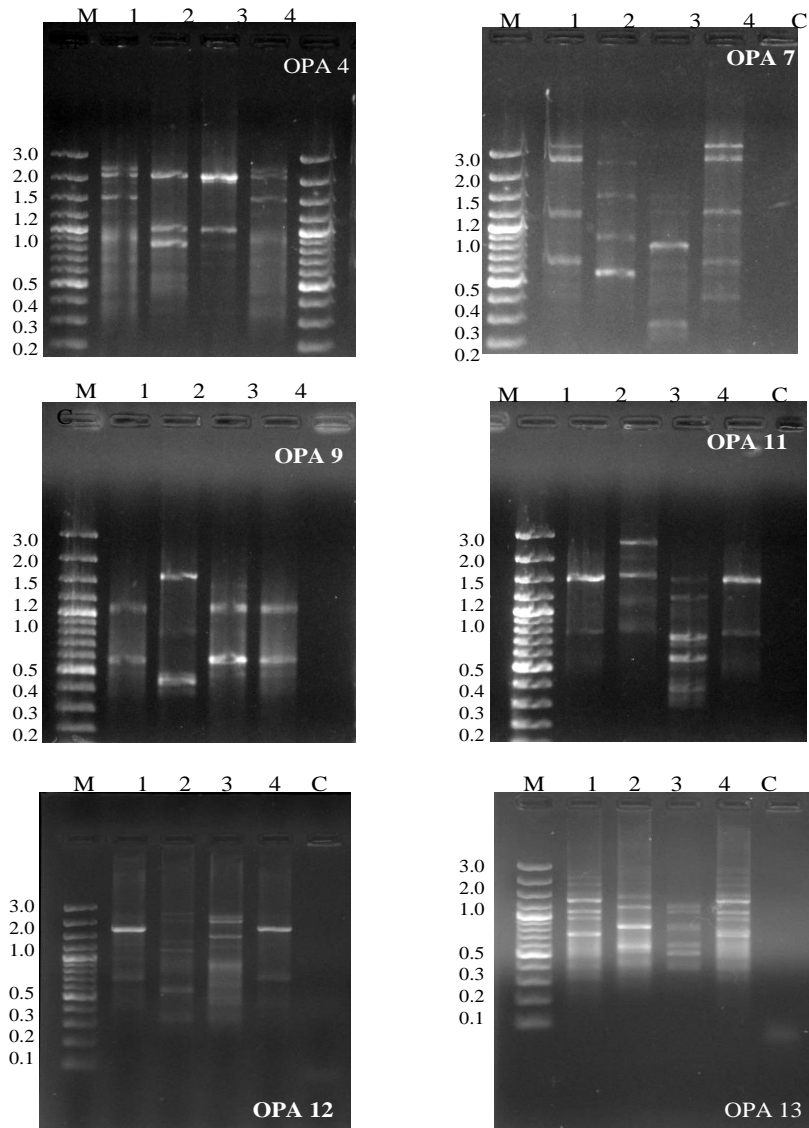


Figure 1. Molecular Variability of four virulent isolates of *A. solani*.

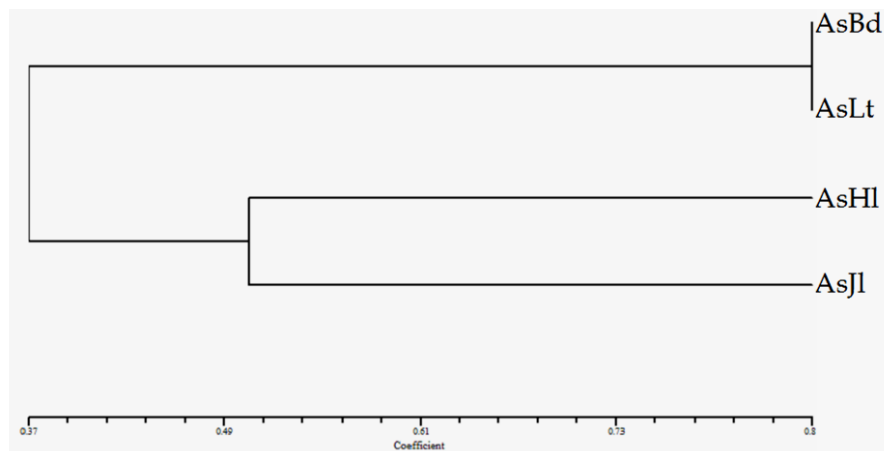


Figure 2. Dendrogram based on RAPD – PCR analysis depicting the relationship among the four fungal pathogens inciting early blight of tomato.

it could be concluded that there may be possibility of the existence of variability amongst *A. solani* isolates in the Marathwada region; however, its further confirmation is must. The future strategy could include collection of large number of samples from different parts of country from tomato, potato and other solanaceous hosts to get as many isolates as possible. This possibly aid in screening tomato and potato genotypes against broad spectrum of pathogen populations towards durable resistance.

Conflict of interests

The authors did not declare any conflict of interest.

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