Mycopath (2017) 15(1): 7-11 **Research Article**

Cultural, morphological, molecular comparison and pathogenicity of *Alternaria solani* causing early blight disease in tomato

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Abstract

Early blight of tomato is one of the highly destructive diseases caused by an airborne soil-inhabiting fungus *Alternaria solani* (Ellis and Martin). Seven *A. solani* strains were isolated from the naturally infected different tomato plants growing in fields and tunnels at Lahore, Kasur, Faislabad, Islamabad, Lalyani Peshawer and vegetable market Lahore, Pakistan. All strains of *A. solani* showed variations in morphological feature. These fungal strains were identified and compared on the basis of morphological and ITS sequence analysis. All strains shared 87.7% homology with genetic distance of 0.174. Pathogenicity test of the isolated strains confirmed their host specificity and re-isolation of the pathogen from symptomatic tomato fruit fulfilled Koch's postulates.

Keywords: Tomato, Early blight, *Alternaria solani*, Virulence

Introduction

Tomato is a nutritious and economically important horticulture crop affected by over 200 pathogens including 24 fungi, 7 bacteria, 10 viruses, 3 viroids and multiple nematodes (Arie *et al*., 2007). However, among fungal pathogens, *Alternaria solani* Ellis and Martin causing early blight disease is responsible for maximum destruction in both growth and yield (Gondal *et al*., 2012, Abdussamee *et al*., 2014). *A. solani* formerly recognized by the name *A. porri* f. sp. *solani* (Neergaard, 1945) is a member division Deuteromycotina (Fungi Imperfecti), class Hyphomycetes and order Hyphales (Agrios, 2005). It is an airborne fungus that can survive in adverse environmental conditions due to presence of pigments like melanin in conidia (Rotem, 1994). In unfavorable environment it overwinters in soil, plant debris, seed and alternate hosts in the form of either conidia or mycelia (Chaerani and Voorrips, 2006). Along with the conidia, it can also harm by producing mycotoxins. So far, 11 toxins including alternariol, altersolanol A, altertoxin, macrosporin and solanapyrone (A, B, and C) and alternaric acid were detected in culture filtrate of the fungus that induce chlorotic and necrotic symptoms (Chaerani and Voorrips, 2006; Andersen *et al*., 2008; Kasahara *et al*., 2010).

Morphologically, it is characterized by multinucleated cells as well as separate dark-coloured, muriform, septate (transverse and longitudinal), beaked conidia, that may be produced either individually or in a chain. (Ellis and Gibson, 1975; Rotem, 1994). However, its identification is under revision after isolation of *A. solani*-like isolates i.e. *Alternaria tomatophila* from Solanaceae hosts (Simmons, 2000) and scientists are now using molecular approaches. Molecular techniques includes the amplification of fungal DNA through PCR by using primers from internal transcribed spacer (ITS) region as well as coding region of the β-tubulin gene and cytochrome b (Pavón *et al*., 2012; Edin *et al*., 2012). ITS primers are specifically used to identify *A. solani* by Zheng *et al* (2015) and Jeon *et al*. (2016) during their studies. In the current study different strains of *A. solani* were isolated and identified on the basis of morphological and molecular attributes.

Materials and Methods

Sample collection

Survey of tomato fields and tunnels at Lahore, Kasur, Faislabad, Islamabad, Lalyani and Peshawer was conducted during February-March, 2014 to collect infected tomato leaves. Typical symptoms of early blight disease i.e. dark brown to black, necrotic lesions surrounded chlorotic halo on leaves, stems and fruits (Gleason and Edward, 2006) were observed**.** The leaves showing early blight disease were collected in sterilized polythene bags, labeled and were brought to the laboratory by placing it on ice in an icebox.

Isolation of *A. solani*

Infected leaves were washed and cut into small pieces of 0.5-1 cm long. Sodium hypochlorite solution (0.5%) was used to surface sterilized leave pieces followed by placing of pieces on 2% Malt extract agar [(MEA) (20 g Malt extract, 20 g agar in 1000 mL^{-1} distilled water)] and Potato dextrose agar [(PDA) (39 g potato dextrose, 20 g agar in1000 mL^{-1} distilled water)] in glass Petri plates

under aseptic conditions. Petri plates were kept at 25 ± 2 °C for 5 days to get pure colonies, which were later sub-cultured to get a single colony plates for identification.

Morphological based identification

Isolated pathogen was initially identified through cultural and microscopic characteristics. Cultural properties like color, zonation, margin and diameter were studied. Microscopically both mycelia and conidia were observed for septation, color, width and shape (Domsch *et al*., 1980). The culture of *A. solani* isolated from the samples obtained from Lahore vegetable market, Kasur, Lahore, Faisalabad, Peshawar, Islamabad and Lalyani were named as Strain 1, Strain 2, Strain 3, Strain 4, Strain 5, Strain 6 and Strain 7, respectively.

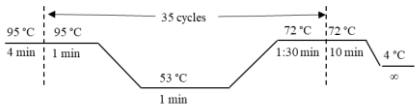
Pathogenicity of the isolated strain was checked by infecting tomato fruits with sterilized inoculating needle in aseptic conditions and placed in an incubator at 25±2℃.

Molecular based identification

Morphology based identification was confirmed by using molecular marker of internal transcribed spacer i.e. ITS1 and ITS4 primers with sequence of 5′-TCCGTAGGTGAACCTGCGG-3′ and 5′-TCCTCCGCTTATTGATATGC-3′, respectively (Loganathan *et al*., 2016).

For genomic DNA isolation, cells of *A. solani* were prepared in 100 mL of 2% ME broth containing 0.1% Tween 80-containing saline. The inoculated flaks were left for 4-5 days at 25 ± 2 °C in a rotating shaker at 100 rpm. Muslin cloth was disinfected with ethanol followed by washing with cold sterilized distilled water was done to harvest the fungal cell. DNA was isolated by grinding 300 mg of fungal cells in liquid nitrogen followed by an incubation of 30 min at 37 °C in Nucleon Reagent B (2 mL) with RNase A (0.5 µL) in a sterile Falcon. Subsequently, 0.5 mL of 5 M $NaClO_4$ and 2 mL of ice chilled chloroform (at -20 °C) were added and mixed vigorously. The tube was then centrifuge at 4000 rpm for 5 min. Supernatant was precipitated with 2 mL of chilled 96% ethanol and centrifuged (4000 rpm) for 5 min. DNA pellet obtained was treated with 70% ethanol to remove protiens followed by air drying. Later, DNA pellet was dissolved in 50 µL TE buffer by incubating for 15 min at 65 °C (Akhtar *et al*., 2014). Isolated DNA was checked by 1% agarose gel electrophoresis.

Quantified DNA was used to carry out PCR at 53 °C with ITS1 and ITS4. Each PCR with total volume of 50 µL reaction mixture was performed in PCR machine (Major sciences, amplification Gene Amp PCR system 25). Reaction mixture comprised of 300 µM each of dATP, dCTP, dGTP, and dTTP, 2.5 mM of $MgCl_2$, 1U Taq DNA polymerase, 5 µL of 10× buffer, 1 µM of each primer, and 2 µL of quantified fungal DNA as template (Foolad *et al*., 1995). The control reaction consisted of sterile, nuclease free water instead of DNA. The PCR machine was programmed as follows:



PCR amplification was checked by performing 1 % agarose W/V gel electrophoresis. PCR product was gene cleaned by using Gene extraction Vivantis kit and were sequenced from Macrogen, Inc. South Korea.

The peaks for individual bases were checked by using MEGA7 v.7.0 and poor or ambiguous bases were not considered in final sequence. The final sequences were nblast at NCBI, submitted to NCBI database and were aligned by multiple sequence alignment of clustal W programme. Cluster analysis was performed to check the homology and phylogeny among the isolated *A. solani* strains by DNAMANv9 software.

Results

The fungal strains generally had concentric zonation with regular and smooth margin, brownish black in color reaching 6-8.8 cm in diameter on MEA after 7 days. Mycelium was septate, hyaline and 0.9-1.6 µm in width. Conidia were hyaline 25-45 µm × 7-10 µm, have both longitudinal (3-10) as well as transverse septa (1-3). Conidial color pale golden or olivaceous brown, beak plate colored sometimes branched (Fig. 1 A-G; Table 1 and 2).

Table 1: Macroscopic features shown by various *A. solani* strains.

A. solani strain	Colony (cm)	Colony color	Zonation
Strain 1	5	Brown to black	-
Strain 2	6.5	Brown to black	-
Strain 3	8.8	Dark brown to black	+
Strain 4	6	Black	-
Strain 5	8.8	Dark brown to black	+
Strain 6	6.5	Dark brown to black	+
Strain 7	7.9	Black	+

Table 2: Microscopic features shown by various *A. solani* strains.

A. solani strain	Mycelium	Conidia			
	Width (µm)	No. of septa		Width (µm)	Beak (µm)
		Longi	Trans		
Strain 1	1.1	3-7	1-2	10-30	20
Strain 2	1.5	5-7	2-3	7-43	33
Strain 3	1.6	4-8	1-2	10-45	50
Strain 4	1.2	4-5	1-2	9-40	45
Strain 5	0.9	3-6	1	9-45	10
Strain 6	1.1	5-9	1-3	8-35	48
Strain 7	1.5	6-10	1-3	10-41	15

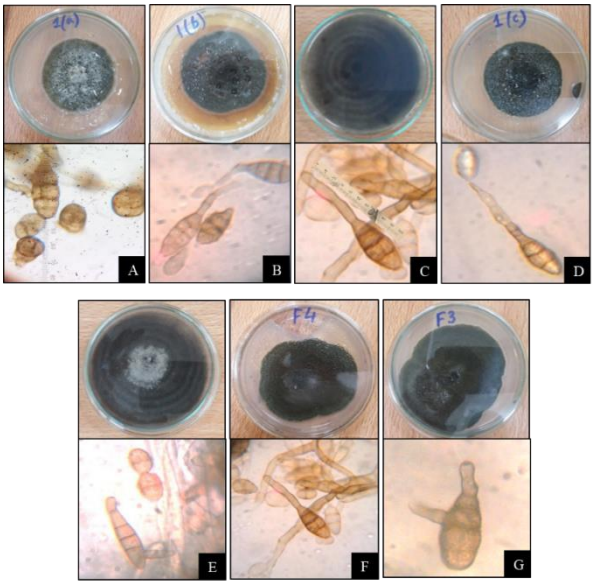


Fig. 1: Macroscopic (Culture plate) and microscopic [Conidia (100 X)] characters of *Alternaria solani*. **A:** Strain 1; **B**: Strain 2; **C:** Strain 3; **D:** Strain 4; **E:** Strain 5; **F:** Strain 6; **G:** Strain 7**.**

The amplified products of all strain documented on 1% agarose gel was about 0.55 kb (Fig. 2) and was sequenced. Sequenced amplicons analyzed by MEGA7 revealed that the sequences were of good quality, noise free, having evenly-spaced peaks each with single color and varying height. The sequences of Strain 1 (532 bp), Strain 2 (527 bp), Strain 3 (518 bp), Strain 4 (531 bp), Strain 5 (551 bp), Strain 6 (549 bp) and Strain 7 (531 bp) were submitted to GenBank under accessions (LC339934, LC339935, MF539619, LC339936, LC339937, LC339938 and LC339939), respectively. BLAST search with the sequences of Stain (1-7) showed 97-100% 99% homology to around 50 different strains of *A. solani* deposited in genebank including different strains (EU315064.1) or isolates (JF491202.1) amplified by ITS primers.

Moreover, the sequences were aligned by using multiple sequence alignment of software DNAMAN v. 9.0. and showed 87.33% identity among *A. solani* strains. The homology dendogram dichotomize *A. solani* sequences fragments into two major divisions (I and II) with overall homology of the 66% (Fig. 3). Division I comprised further 2 subdivision i.e. IA and IB with 99% identity. IA consisted of the nucleotide fragments amplified *A. solani* strain (1, 2, 4, 7 and 6) and IB contained only *A. solani* strain 3. Group II included *A. solani* strain 5 (Fig. 3). Similarly, the phylogenetic tree based on 0.176 genetic distances segregate *A. solani* strain sequences into two major groups i.e. group I and group II. Group I consist *A. solani* strain (1, 4, 7, 3, 6 and 2). Whereas, Group II comprised of only *A. solani* strain 5 (Fig. 4).

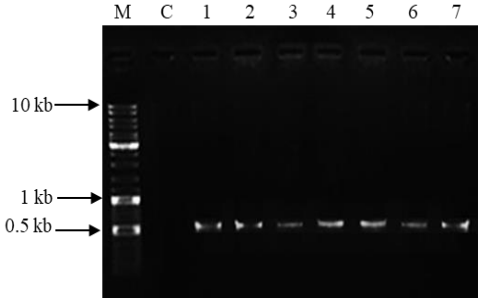


Fig. 2: PCR product of ITS (1 and 4) with *A. solani* strains on 1 % agarose gel electrophoresis **M:** Ladder #SM0331**; 1:** Strain 1; **2:** Strain 2; **3:** Strain 3; **4:** Strain 4; **5:** Strain 5; **6:** Strain 6; **7** Strain. 7.

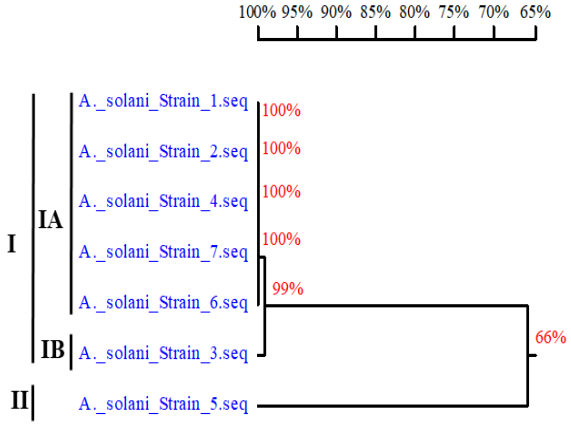


Fig. 3: A homology dendogram made by using DNAMAN v.9 showing the alignment of ITS (1 and 4) sequences obtained from Strain 1, Strain 2, Strain 3; Strain 4; Strain 5; Strain 6 and Strain 7.

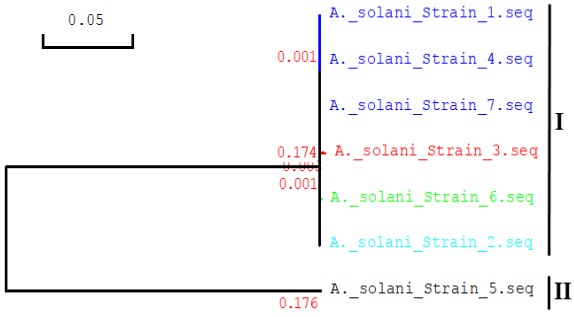


Fig. 4: A phylogentic dendogram of amplified sequences by primer pair ITS (1+4) obtained from Strain 1, Strain 2, Strain 3; Strain 4; Strain 5; Strain 6 and Strain 7.

Pathogenicity of the *A. solani* stains were checked by inoculating fresh tomato fruits. The strain 3 and 4 were considered as virulent. As both strains showed the severe and typical symptoms of early blight disease i.e. Dark brown to black lesions with smooth margin and chlorotic halo that finally rotten the fruits (Fig. 5).

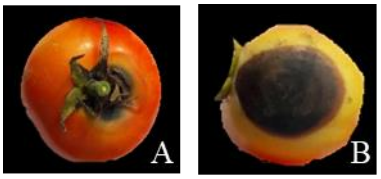


Fig. 5: Tomato fruits showing symptoms of early blight disease infected by *A. solani* strains.
A: Strain 3; **B:** Strain 4.

Discussion

All strains of *A. solani* isolated and studied showed variations in morphological feature that is common among the specie (Barksdale, 1969). Morphological characteristics like margins were regular and smooth in all seven strains (Ellis and Gibson, 1975). The findings of the present study were concurrent with results of previous investigations on the basis of cultural characteristics among various strains of *A. solani* (Babu *et al*., 2000; Naik *et al*., 2010). However, mycelia color, growth and zonation pattern were different. These characteristics are less likely to be affected by media but mainly depend on the strain/isolate (Kumar *et al*., 2008; Singh *et al*., 2014; Nikam *et al*., 2015). Microscopic features including mycelia and conidia were studied. Mycelia were septate, hyaline and of 0.9-1.6 µm. Conidia were hyaline 25-45 µm × 7-10 µm, septate (longitudinal and transverse) and beaked (50 µm long). Similar results were found by Simmons (2000) and Ahmad (2002). The difference in spores sizes showed the similarity with larges pored Alternaria species (Van der Waals *et al*., 2001).

All strains amplified the product of about 0.55 kb with primers ITS1 and ITS4. Previously, different *A. solani* species generated band size ranges from 400–600 bp through internal transcribed spacer (ITS) regions primers (Pryor and Gilbertson, 2000; Manter and Vivanco, 2007; Embong *et al*., 2008). Moreover, homology and phylogenetic analysis among *A. solani* strains assessed by the sequences produced close proximity. Maximum homology was observed among strains i.e. 1, 2, 3, 4, 7 and 6 whereas, strain 5 is least similar. The phylogenetic tree basedalso gave the similar results. Similar results with Alternaria species were recorded in former studies (Berbee, 1996; Pryor and Gilbertson, 2000).

A. solani strains 3 and 4 produced severe symptoms of necrotic lesions (dark brown to black color) that resulted in the rotting of tomato fruits. Same symptoms on fruits were also observed in earlier investigations (Chaerani *et al.*, 2007; Gleason and Edward 2006; Blancard *et al*., 2012)

References

Abdussamee H, Hussain M, Ali M, Siddique MS, Khan SU, 2014. Genetic response of tomato germplasm against early blight and its management through fungicides. *Appl. Sci. Rep.*, **2**: 119-127.

Agrios GN, 2005. Plant pathology (5th ed.): New York: Academic Press, New York.

Ahmad S, 2002. Conidial morphology of Alternaria solani, its variations in tomato leaf blight. *Ann. Agric. Res.*, **23**: 514-515.

Akhtar N, Shoaib A, Munir S, Ali A, Khurshid S, 2014. Isolation, identification and enzyme production profile of *A. niger*. *J. Anim. Plant Sci.*, **24**: 1438-1443.

Andersen B, Dongo A, Pryor BM, 2008. Secondary metabolite profiling of *Alternaria dauci, A. porri, A. solani, and A. tomatophila. Mycol. Res.*, **112:** 241-250.

Arie T, Takahashi H, Kodama M, Teraoka T, 2007. Tomato as a model plant for plant-pathogen interactions. *Plant Biotechnol.*, **24:** 135-147.

Babu S, Seetharaman K, Nandakumar R, Johnson I, 2000. Variability in cultural characteristics of tomato leaf blight pathogen. *Plant. Dis. Res.*, **15:** 121.

Barksdale TH, 1969. Resistance of tomato seedlings to early blight. *Phytopathology*, **59:** 443-446.

Berbee ML, 1996. Loculoascomycete origins and evolution of filamentous ascomycete morphology based on 16S rDNAgene sequence data. *Mol. Biol. Evol.*, **13:** 462–470.

Blancard D, Laterrot H, Marchoux G, Candresse T, (2012) A colour Handbook - Tomato Diseases: identification, biology and control. Manson Publishing Manson Publishing Limited London UK: pp. 688.

Chaerani R, Groenwold R, Stam P, Voorrips RE 2007. Assessment of early blight (*Alternaria solani*) resistance in tomato using a droplet inoculation method. *J. Gen. Plant Pathol.*, **73**: 96-103.

Chaerani R, Voorrips RE, 2006. Tomato early blight (*Alternaria solani*): the pathogen, genetics, and breeding for resistance. *J. Gen. Plant Pathol.*, **72:** 335–347.

Domsch KH, Gams W, Anderson TH, 1980. Compendium of soil fungi. Academic Press, London.

Edin E, 2012. Species specific primers for identification of *Alternaria solani*, in combination with analysis of the F129L

substitution associates with loss of sensitivity toward strobilurins. *Crop Prot.*, **38:** 72-73.

Ellis MB, Gibson IAS, 1975. *Alternaria solani*. CMI Descriptions of Pathogenic Fungi and Bacteria No. 475.

Embong Z, Hitam WH, Yean CY, Rashid NH, Kamarudin B, Abidin SK, Osman S, Zainuddin ZF, Ravichandran M, 2008.Specific detection of fungal pathogens by 18S rRNA gene PCR in microbial keratitis. *BMC Ophthalmol.,* **8**: 7.

Foolad MR, Arulsekar S, Rodriguez RL, 1995. Applications of polymerase chain reaction (PCR) to plant genome analysis. In: Fundamental Methods of Plant Cell Tissue and Organ Culture and Laboratory. Springer International Berlin, pp. 281-298.

Gleason ML, Edmunds BA, 2006. Tomato diseases and disorders: Iowa State University, University Extension. United States.

Gondal AS, Ijaz M, Riaz K, Khan AR, 2012. Effect of different doses of fungicide (Mancozeb) against alternaria leaf blight of tomato in tunnel. *J. Plant Pathol. Microb.*, **3**: 125.

Jeon CW, Hong SW, Cho H, Kwak YS, 2016. *Alternaria solani* causing leaf blight disease on *Aster glehni* in Korea. *J. Agric. Life Sci.*, **50**: 249-253.

Kasahara K, Miyamoto T, Fujimoto T, Oguri H, Tokiwano T, Oikawa H, Ebizuka Y, Fujii I, 2010. Solanapyrone Synthase, a Possible Diels-Alderase and Iterative Type I Polyketide Synthase Encoded in a Biosynthetic Gene Cluster from *Alternaria solani*. *Chem. Bio. Chem.*, **11:** 1245-1252.

Kumar V, Haldar S, Pandey KK, Singh RP, Singh AK, Singh PC, 2008. Cultural, morphological, pathogenic and molecular variability amongst tomato isolates of *Alternaria sola*ni in India. *World J. Microbiol. Biotechnol.*, **24:** 1003-1009.

Loganathan M, Venkataravanappa V, Saha S, Rai AB, Tripathi S, Rai RK, Pandey AK, and Chowdappa P, 2016. Morphological, pathogenic and molecular characterizations of *Alternaria* species causing early blight of tomato in Northern India. *Proc. Natl. Acad. Sci. USA Indian Sec. B: Biol. Sci.* **86:** 325-330.

Manter DK, Vivanco JM, 2007. Use of the ITS primers, ITS1F and ITS4, to characterize fungal abundance and diversity in mixed-template samples by qPCR and length heterogeneity analysis. *J. Microbiol. Methods*, **71**: 7-14.

Naik MK, Prasad Y, Bhat KV, Rani GSD, 2010. Morphological, physiological and molecular variability among isolates of *A. solani* from tomato. *Indian Phytopathol*., **63:** 168-173.

Neergaard P, 1945. Danish species of *Alternaria* and *Stemphylium*. Taxonomy, parasitism, economical significance. Copenhagen: Munksgaard: Oxford University Press.

Nikam PS, Suryawanshi AP, Chavan AA, 2015. Pathogenic, cultural, morphological and molecular variabilty among 8 isolates of *A solani* causing early blight of tomato. *Afr. J. Biotechnol.*, **14**: 872-877.

Pavón MÁ, Luna A, de la Cruz S, González I, Martín R, García T, 2012. PCR-based assay for the detection of *Alternaria* species and correlation with HPLC determination of altenuene, alternariol and alternariol monomethyl ether production in tomato products. *Food Control*, **25**: 45-52.

Pryor BM, Gilbertson RL, 2000. Molecular phylogenetic relationships amongst Alternaria species and related fungi based upon analysis of nuclear ITS and mt SSU rDNA sequences. *Mycol. Res.*, **104:** 1312-1321.

Rotem J, 1994. The genus Alternaria. St. Paul: APS Press.

Simmons EG, 2000. *Alternaria* themes and variations (244-286) species on Solanaceae. *Mycotaxon*, **75**: 1-115.

Singh A, Singh V, Yadav SM, 2014.Cultural, morphological and pathogenic molecular variabilty of *A solani* causing early blight of tomato. *Plant Pathol. J.*, **13:** 167-172.

Van der Waals JE, Korsten L, Aveling TAS, 2001. A review of early blight of potato. *Afr. Plant Prot.*, **7**: 91-102.

Yadav S, Singh V, Chand R, 2015. Mass sporulation of *Alternaria solani* causing early blight of tomato. *Indian Phytopathol.*, **68**: 83-86.

Zheng HH, Zhao J, Wang TY, Wu XH, 2015. Characterization of Alternaria species associated with potato foliar diseases in China. *Plant Pathol*., **64**: 425-433.