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FIRST REPORT OF *Alternaria metachromatica* FROM PAKISTAN CAUSING LEAF SPOT OF TOMATO

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Tomato (*Lycopersicon esculentum* Mill.) is one of the most popular and significant commercial crop of Pakistan. Outbreak of leaf spot was observed on leaves of tomato plants growing in fields of Lahore. Causal organism was isolated and initially identified on the basis of morphological characters. Complete description of macro and microscopic characters was prepared. Identification was confirmed by rDNA spacer sequence. Based on morphological and molecular approaches, *Alternaria metachromatica* was identified as causal organism. Culture of pathogen was deposited in First Fungal Culture Bank of Pakistan (FCBP) and the gene sequence in GenBank. Pathogenicity was confirmed by exposing the healthy leaves to spores of *A. metachromatica* as well as inoculums in the soil.
Keywords: Leaf spot, *Alternaria*, rDNA, morphological characters

INTRODUCTION

Tomato (*Lycopersicon esculentum* L.),an important vegetable and model research organism is grown throughout the world (Naika *et al.,* 2005; Kimura and Sinha, 2008).This crop is prominent for its nutritional value. Fruit of this plant is rich in lycopene, β-carotene, carotenoids, vitamin C, potassium and fiber (Rani and Khetarpaul, 2009; Britt and Kristin, 2011).

Being saprophytes as well as plant pathogens, species of genus *Alternaria* are widely distributed in our environment. Members of this group are known to cause spots, rots and blights in plants (Agrios, 2005). Identification of *Alternaria* species is very complex. Simmons (2007) provided the detailed manual for *Alternaria* identification.

Alternaria species are major plant pathogens, which cause at least 20% of agricultural spoilage; most severe losses may reach up to 80% of yield, affecting the leaves, stems, flowers and fruits (Nowicki *et al.,* 2012). *Alternaria* black spots are usually brown or black present on leaves, stems or pods that enlarge under warm and humid environmental conditions resulting in reduced photosynthetic area, defoliation and accelerated senescence (Conn *et al*., 1988). In Pakistan, *Alternaria* black spot disease is very common and has been reported on a wide range of hosts for example sunflower (Mirza and Beg, 1983), tomato (Akhtar *et al*., 2004), *Aloevera* (Bajwa *et al*., 2010) and Mango (Mohsan *et al*., 2011). Present study reports a new pathogen of tomato leaf spot from Pakistan.

MATERIALS AND METHODS

***Morphological identification*:** The *Alternaria* species was identified according to the identification key proposed by Simmons (2007). Morphological studies were carried out on the strains grown at 25°C on malt extract agar (MEA) and potato carrot agar (PCA) media. Colony characters were observed using stereo light microscope as; color of culture and reverse, number of growth zones, diameter of colony (cm), presence of aerial and submerged mycelium, type of conidial chains and abundance of conidia.

The microscopic characteristics to identify the *Alternaria* species were; color, shape, number and position of septa (longitudinal, oblique ortransverse) of conidia and their attachment with the conidiophores, ornamentation of conidial walls, presence, size and shape of conidial beak, presence of apical or basal pores.

***Molecular analysis*:** Identification was confirmed by means of Internal Transcribe Spacer sequence. The genomic DNA of fungal species studied was extracted with CTAB method (Weigand *et al*., 1993). Quality of extracted DNA was checked by gel electrophoresis on 0.8% agarose containing 10 mg/ml ethidium bromide along with DNA size marker.

The Beer-Lambert Law, i.e. the linear relationship between absorbance and the concentration of a sample at a specific wavelength of light was used to calculate the concentration of DNA samples. DNA was quantified by following the equation

$$A = \varepsilon \times l \times c$$

Where A is absorbance, ε is the coefficient of absorbance, l is the path length, and c is the concentration. The concentration of the genomic DNA in all samples was adjusted to 25-50 ng/µl by diluting with TE buffer.

The internal transcribed coding regions of genome were amplified using the genomic DNA as template (White *et al.,* 1990). Taq polymerase with appropriate buffer was used for amplification in a 25 µl PCR reaction mixture (1 µl of DNA, 0.2mM dNTPs, 0.5 µM of each primer, ITS1 forward (5′-TCC GTA GGT GAA CCT GCG G -3′) and ITS4 reverse

primer (3´- TCC TCC GCT TAT TGA TAT GC-5´) and 0.5 u of Taq polymerase). PCR reaction was carried out according to the following programme; one cycle at 95°C for 90 sec followed by 30 cycles each of denaturation at 95°C for 10 sec, annealing at 60°C for 20 sec elongation at 60°C for 10 sec. PCR product was run on 1% agarose gel and visualized by UV light. Amplified DNA was sent for sequencing. DNA sequence results were analysed employing different bioinformatics tools.

***Pathogenicity test*:** Fungal pathogenicity was confirmed by growing the tomato plants in pathogen infected soil as well as spray of fungal spores onto the leaves of young tomato plants. A suspension of fungal spores containing 10^5 spores per ml was prepared. Spore suspension (50 ml) was mixed well with sterilized sieved 500 g of soil. This soil was filled in each plastic pots followed by sowing of 5 seeds per pot. Plants were grown in at 25°C and relative humidity 90-100%. Plants were regularly monitored for disease development.

RESULTS

***Identification of pathogen*:** *Colony* on MEA at 25°C reached to 2.5-3.5 cm in diameterin 6-7 days (Fig. 1 A-B) radiate, floccose, 2-3 concentric rings after 2 weeks. Colony color was olivaceous green to black; reverse greenish black, exudates and odor not present. Colony grew restrictedly on PCA at 25°C, 1-1.5 cm in diameter (Fig. 1 C-D), radiate, floccose, concentric rings formation not clear.

Conidial chains with several short lateral branches of 5-7 conidia; *Conidiophores* abundant, branched and short, emerged directly from agar surface; simple chain of 8-10 conidia; secondary conidiophores not conspicuously long, 20-30 x 3-4 µm, 1-2 geniculate. *Conidia* of all ages were ovoid and beak was present in some condia, while absent in others. Mature conidia reached in size of 25-45 x 8-12 µm, with 4-7 transverse and 1-2 longitudinal septa.

Based on morphology, pathogen was identified as *Alternaria metachromatica* (Simmons, 2007).Pure culture of this strain was deposited in First Fungal Culture Bank of Pakistan (FCBP) under the accession number FCBP1350 which can be obtained from FCBP for teaching and research purposes.

***Molecular analysis*:** The consensus primers ITS1 and ITS4 were used for the amplification of Internal transcribed region. Fungus-specific universal primer pairs (ITS1 and ITS4) were able to successfully amplify the ITS1–5.8S

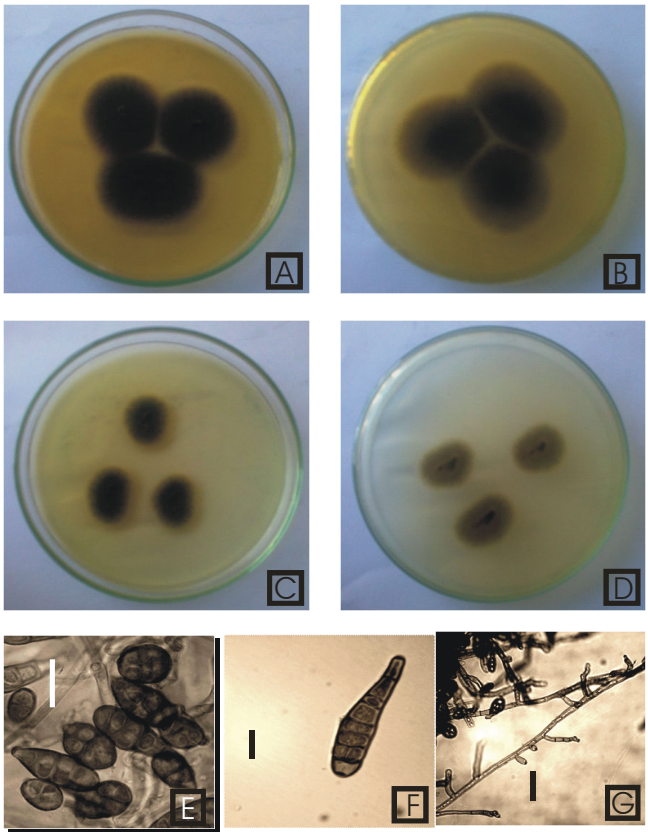

Figure 1. ***Alternaria metachromatica*; A-B: Colony and reverse on MEA, C-D: colony and reverse on PDA, E-F: conidia, G: conidiophores**

rDNA-ITS4 region of *A. metachromatica*. A single PCR product of about 650 bp was observed on a 1% agarose gel (Fig. 2). Purified PCR products yielded sequence of 533bp in length, that was deposited to GenBank under the accession number KF496083 (Fig. 3).Using the National Center for Biotechnology Information (NCBI) and European Bioinformatics Institute (EBI) bioinformatics websites, DNA sequences were blast.

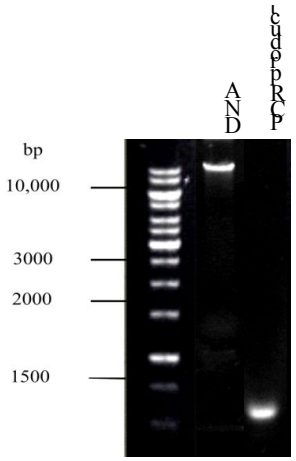

Figure 2. Genomic DNA isolated and amplified Internal Transcribed Spacer (ITS) sequence from genomic DNA of pathogen.

Blast searches revealed that the ITS nucleotide sequence was 100% identical with *A. metachromatica,* isolate K2 (KF772936.1), causing infection of tobacco seeds China. The nucleotide homology of ITS sequences of both *A. metachromatica* isolates (FCBP 1350 and K2) is represented in Fig.4.

***Confirmation of Koch's postulate*:** Identical disease symptoms appeared after 10 days of spore inoculation whereas control plants remained healthy. *A. metachromatica* was re-isolated from diseased leaves. Results of pathogenicity test fulfilled Koch's postulate.

***Conclusion*:** To the best of our knowledge this is not only the first report of *A. metachromatica* leaf spot of tomato but also addition of a new species of Genus *Alternaria* to the record of fungi of Pakistan.

>gi|532809693|gb|KF496083.1| *Alternaria metachromatica* strain FCBP1350 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
AAAACCTCCGTATGATGCGGGCTGGACCTCTCGGGGTTACAGCCTTGCTGAATTATTCAC
CCTTGTCTTTTGCGTACTTCTTGTTTCCTTGGTGGGTTCGCCCACCACTAGGACAAACATA
AACCTTTTGTAATTGCAATCAGCGTCAGTAACAAATTAATAATTACAACTTTCAACAACG
GATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAGTGTGAATT
GCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCTTTGGTATTCCAAAG
GGCATGCCTGTTCGAGCGTCATTTGTACCCTCAAGCTTTGCTTGGTGTTGGGCGTCTTGTC
TCTAGCTTTGCTGGAGACTCGCCTTAAAGTAATTGGCAGCCGGCCTACTGGTTTCGGAGC
GCAGCACAAGTCGCACTCTCTATCAGCAAAGGTCTAGCATCCATTAAGCCTTTTTTTCAA
CTTTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCATATCAAAA

Figure 3. Internal Transcribed Spacer Sequence of *A. metachromatica* submitted to GenBank (KF496083).

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FCBP1350    AAAACCTCCGTATGATGCGGGCTGGACCTCTCGGGGTTACAGCCTTGCTGAATTATTCAC  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
K2          AAAACCTCCGTATGATGCGGGCTGGACCTCTCGGGGTTACAGCCTTGCTGAATTATTCAC  60

FCBP1350    CCTTGTCTTTTGCGTACTTCTTGTTTCCTTGGTGGGTTCGCCCACCACTAGGACAAACAT  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
K2          CCTTGTCTTTTGCGTACTTCTTGTTTCCTTGGTGGGTTCGCCCACCACTAGGACAAACAT  120

FCBP1350    AAACCTTTTGTAATTGCAATCAGCGTCAGTAACAAATTAATAATTACAACTTTCAACAAC  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
K2          AAACCTTTTGTAATTGCAATCAGCGTCAGTAACAAATTAATAATTACAACTTTCAACAAC  180

FCBP1350    GGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAGTGTGAATT  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
K2          GGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAGTGTGAATT  240

FCBP1350    GCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCTTTGGTATTCCAAAG  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
K2          GCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCTTTGGTATTCCAAAG  300

FCBP1350    GGCATGCCTGTTCGAGCGTCATTTGTACCCTCAAGCTTTGCTTGGTGTTGGGCGTCTTGT  360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
K2          GGCATGCCTGTTCGAGCGTCATTTGTACCCTCAAGCTTTGCTTGGTGTTGGGCGTCTTGT  360

FCBP1350    CTCTAGCTTTGCTGGAGACTCGCCTTAAAGTAATTGGCAGCCGGCCTACTGGTTTCGGAG  420
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
K2          CTCTAGCTTTGCTGGAGACTCGCCTTAAAGTAATTGGCAGCCGGCCTACTGGTTTCGGAG  420

FCBP1350    CGCAGCACAAGTCGCACTCTCTATCAGCAAAGGTCTAGCATCCATTAAGCCtttttttCA  480
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
K2          CGCAGCACAAGTCGCACTCTCTATCAGCAAAGGTCTAGCATCCATTAAGCCTTTTTTTCA  480

FCBP1350    ACTTTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCATATCAAAA  533
            ||||||||||||||||||||||||||||||||||||||||||||||||||||
K2          ACTTTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCATATCAAAA  533
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Figure 4. Alignment of *A. metachromatica* isolates FCBP1350 and K2 showing that ITS nucleotide sequences of both isolates are 100% similar.

REFERENCES

Agrios, G.N. 2005. Plant Pathology, Academic Press, New York.

Akhtar, K.P., M.Y. Saleem, M. Asghar and M.A. Haq. 2004. New report of *Alternaria alternata* causing leaf blight of tomato in Pakistan. New Dis. Rep. 9: 43.

Bajwa, R., M. Irum and S. Mushtaq. 2010. New report of *Alternaria alternata* causing leaf spot of *Aloe vera* in Pakistan. Can. J. Plant Pathol. 32: 490-492.

Britt, B.F. and R. Kristin. 2011. Tomato consumption and health: Emerging benefits. Am. J. Life Style Med. 8: 182-191.

Conn, K.L., J.P. Tewari and J.S. Dahiya. 1988. Resistance to *Alternaria brassicae* and phytoalexins-elicitation in rapeseed and other crucifers. Plant Sci. 56: 21-25.

Kimura, S. and N. Sinha. 2008. Tomato (*Solanum lycopersicum*): A model fruit-bearing crop. In: Emerging Model Organisms: A laboratory Manual, Vol. 1. Cold Spring Harbor Laboratory Press, New York.

Mirza, M.S. and A. Beg. 1983. Diseases of sunflower in Pakistan in 1982. Helia 6: 55-56.

Mohsan, M., M. Intizar-ul-Hassan and L. Ali. 2011. Chemotherapeutic management of *Alternaria* black spot (*Alternaria alternata*) in mango fruits. J. Agric. Res. 49: 499-506.

Naika, S., J. Juede, M. Goffau, M. Hilmi and V. Dam. 2005. Cultivation of tomato production, processing and marketing, Agromisa/CTA. Revised edition, Agrodok-series No 17.

Nowicki, M., M. Nowakowska, A. Niezgoda and E.U. Kozik. 2012. Alternaria black spot of crucifers: symptoms, importance of disease, and perspectives of resistance breeding vegetable crops. Res. Bull. 76: 5-19.

Rani, V. and N. Khetarpaul. 2009. Nutrient composition of tomato products prepared using tomato grown under sodic condition with gypsum and farmyard manure treatment. J. Sci. Food Agric. 89: 2601-2607.

Simmons, E.G. 2007. Alternaria: An Identification Manual. CBS, Fungal Biodiversity Center Utrercht, The Netherlands.

Weigand, F., M. Baum and S. Udupa. 1993. DNA molecular marker techniques. Technical Manual No. 20. International Center for Agricultural Research in the Dry Area. Aleppo, Syria.

White, T.J., T. Bruns and S. Lee. 1990. Amplification and Direct Sequencing of Fungal Ribosomal RNA Genes for Phylogenetics. In: J. Taylor, A. Innis, D.H. Gelfand and J.J. Sninsky (eds.), PCR Protocols. Academic Press, San Diego, CA, USA; pp.315-322.