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Short Communication

# OVEL REPORT OF ROSA INDICA LEAF SPOT BY ALTERNARIA TENUISSIMA FROM PAKISTAN

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## ABSTRACT

Fungal leaf spot disease is a major threat to *Rosa indica* in Pakistan. To meet the demands of rose flower, large scale production of roses without any fungal disease is necessary. For this purpose, isolation and identification of pathogen from rose plant was carried out by observing phenotypic characters. Further detailed confirmation of identity of the isolated pathogen was executed on molecular bases by amplification of partial ITS, EF and GAPDH genes and their BLAST analysis. On these bases, fungal pathogen was confirmed as *Alternaria tenuissima*. Finally, Koch's pathogenicity test was applied to ratify the virulence level of novel isolated fungal pathogen by artificially inoculating it on *R. indica* seedlings in Petriplate and pot trials.

**Keywords**: *Alternaria tenuissima,* Identification, leaf spot, pathogenicity, *Rosa indica.*

## INTRODUCTION

Rose plants are type of shrubs with the climbing stem and prickles, have diversity in color and also vary in size of flower. Rose is attacked by various pathogens like bacteria, viruses or fungi during their season of growth, pre-harvesting along with handling and transportation with post-harvest storage and marketing circumstances (Tzanetakis *et al.,* 2006). Alternaria leaf spot disease is considered as the most destructive and damaging fungal diseases to a wide range of hosts. Therefore, this work aimed to study and identify the prevalence of *Alternaria tenuissima* which causes disease on previously non-host plants.

The survey was conducted during August – September (2018) for the collection of infected leaves of rose plants. Different regions of Lahore were selected for collection of diseased samples.

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During survey clear symptoms like dark brown to black spots on the leaves, lesions and a number of necrotic spots were noticed. The sizes of the spots were 2-3 mm and about 50-60% areas of leaves were found to be infected with disease (Figure 1).

For isolation of fungal pathogen, at least 3-4 spots from infected leaves were cut into 3 $mm^2$ small pieces and sterilized using 1% sodium hypochlorite solution and washed with distilled water. Then 4-5 small leaf portions were aseptically inoculated onto Malt Extract Agar plates and incubated at 25 °C. Isolated pathogen from the diseased samples was studied on the basis of morphology and nucleotide sequence analysis. Morphology covered the appearance and microscopic examination of fungal pathogens isolated from diseased samples, whereas sequence alignment of the pathogen was carried out with selected primers to confirm the pathogen.

The rapid growth pattern of *A. tenuissima* colony was observed in MEA medium, at 25-26 °C. The colony size was measured about 4-5 cm in diameter after 4-5 days of incubation. The texture of colony was loose cottony, dark brownish that converted to black at maturity from front side of plate. While a zone of light yellow to golden color

appeared at reverse side of the plate (Figure 2a-b). Under microscope, septate hyphae with 3~5 longitudinal septa and 1-4 diaphragms were noticed. The branched conidiophore of moderate length were found 20-30 × 7-10 µm in size. Conidia were branched or unbranched with varying length of chains (6-12 µm). Conidia had long beak with tapered to ovoid end. Conidia color was brown to black with smooth surface walled, globose and rounded with average size of 4-6 µm (Figure 2c-f) (Shafique *et al*., 2019).

Figure 1. Survey of *Rosa indica* fields of Lahore; (A): Field view, (B): Diseased Rose plant, (C): Foliar disease symptoms.

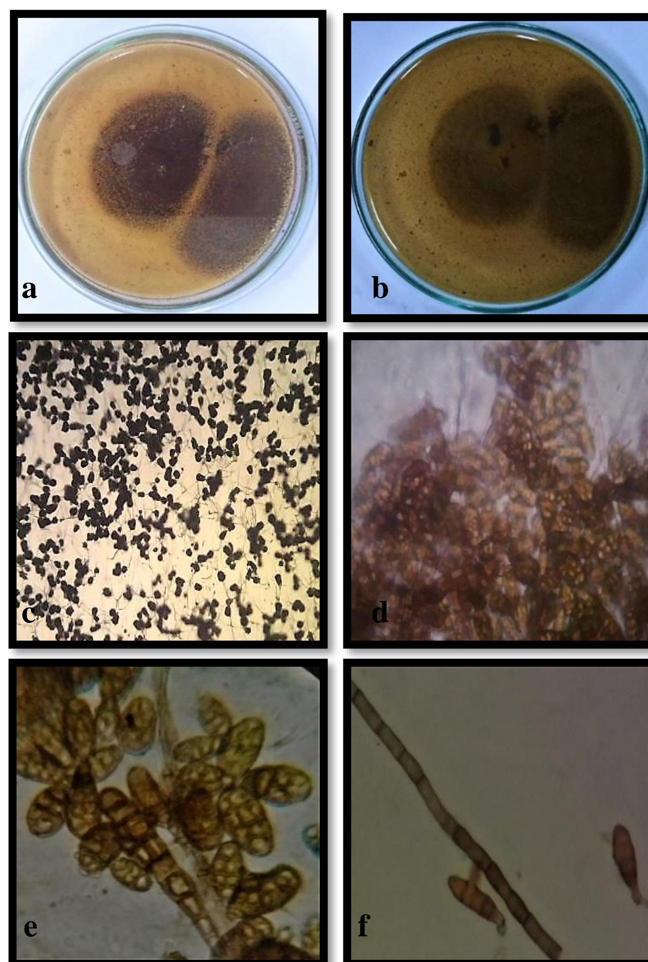


Figure 2. *Alternaria tenuissima* (a): Front and (b) reverse of colony grown on MEA; (c): Mycelia and Conidial heads under stereoscope; and (d-f): Microphotographs of mycelia, conidiophore and conidia at 10X, 40X and conidia at 100X magnification of microscope, respectively.

Genetic characterization of fungal pathogen was concluded by isolation of DNA (Shafique *et al*., 2019). For nucleotide sequencing, the isolated DNA of fungal culture was used in PCR amplifications by internal transcribe sequence spacer (ITS), Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and elongation factor (EF) primers. Amplification impressions revealed that all the PCR products of ITS region of pathogen resulted in the nucleotide sequences of 500-600 bp on agarose gel. The sequences of GAPDH region were 600-700 bp, and EF sequences were 200-300bp in length. Nucleotide sequences were further analyzed and BLAST using National Center for Biotechnology Information (NCBI) website. The identification of fungal species was confirmed on the similarity basis of Blast sequences (99-100%).

The ITS sequence alignment of *A. tenuissima* showed 100% homology with MH790286.1 and 99.74% and 99.49% with the JX406526.1 and MG214858.1 (Figure 3a). The band of 551 bp size was shown on 1% agarose gel. The amplified ITS nucleotide sequence of *A. tenuissima* was assigned MN544937 accession ID in GenBank. *A. tenuissima* with GAPDH sequence of BLAST results showed 99.48% similarity with the *A. tenuissima* KY290574.1 and JN634820.1 (Figure 3b), and portrayed the single PCR product of 594 bp on 1% agarose gel. Translation elongation factor (EF) sequence of *A. tenuissima* found 100% similarity with *A. tenuissima* LT707524.1, MG013473.1 and LC136864.1 (Figure 3c) with 256 bp band. The evolutionary study was directed in MEGA 6 (Tumara *et al*., 2013). The Jukes- Cantor model was used to infer evolutionary history (Jukes and Cantor, 1969).

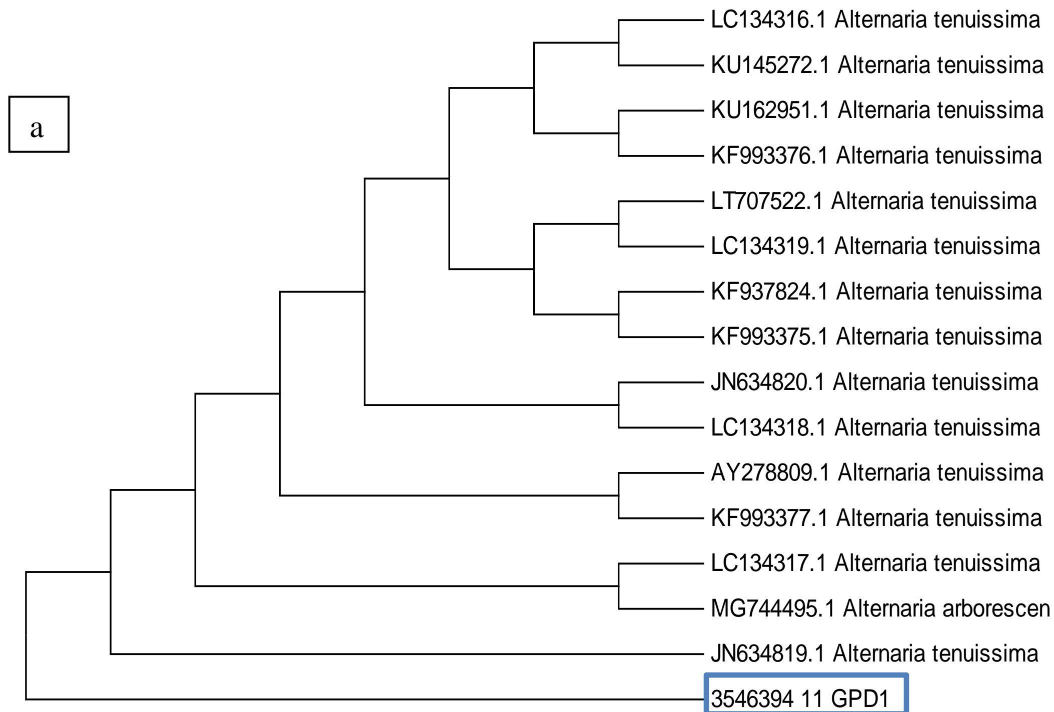


Figure 3. Phylogenetic tree of (a): Internal transcribed spacer region.

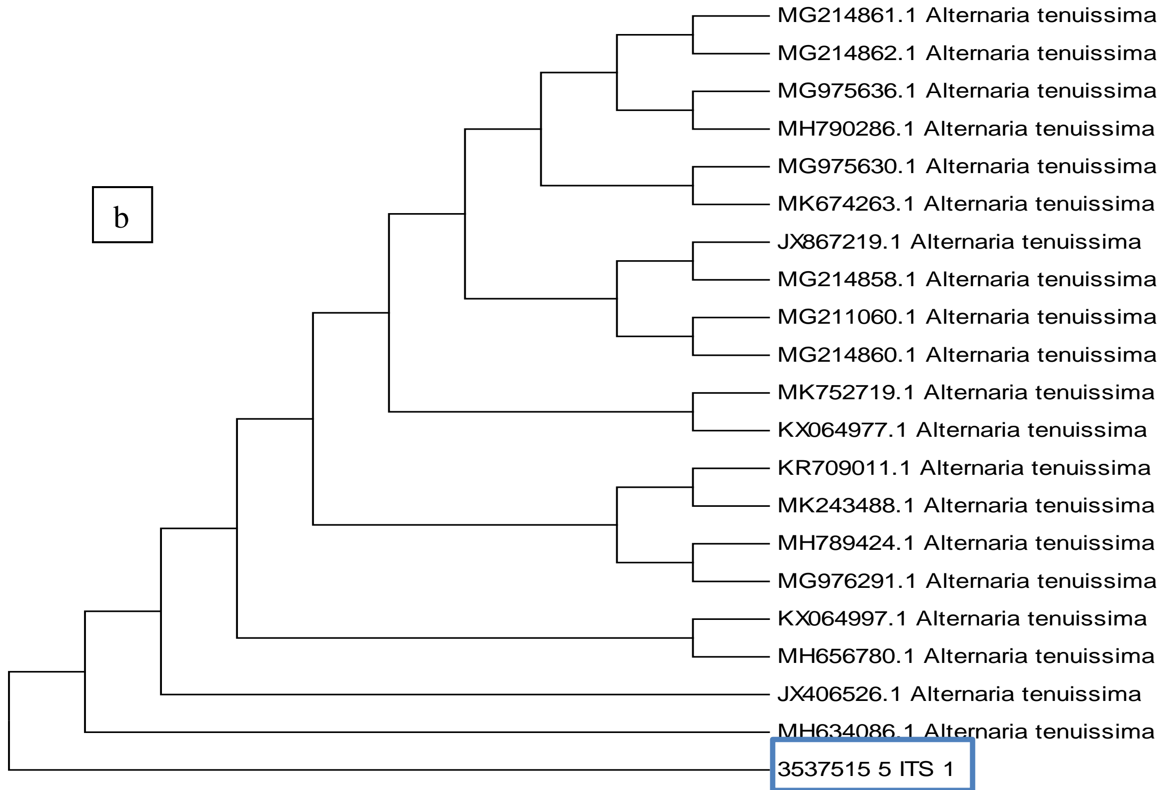


Figure 3. Phylogenetic tree of (b): GAPDH region.

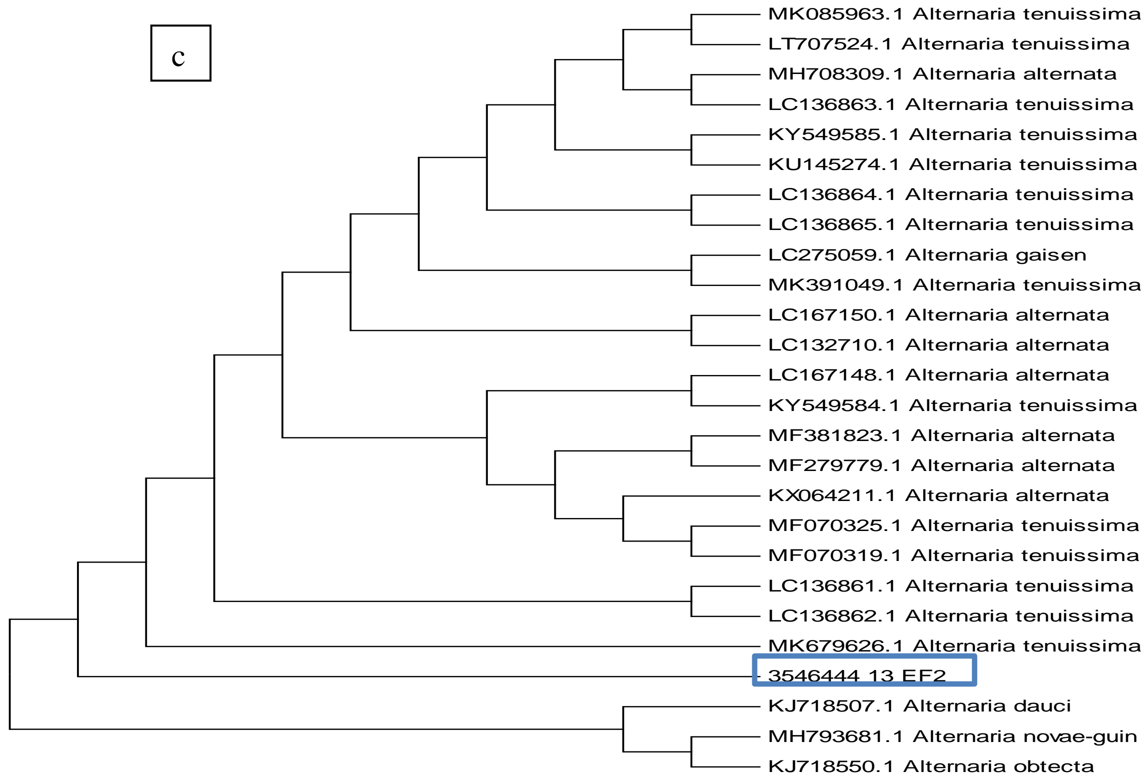


Figure 3. Phylogenetic tree of (c): translation elongation factor (EF) region of rDNA of *A. tenuissima*. MEGA 6 and Jukes-Cantor model was used for evolutionary analysis with maximum likelihood method.

The host specificity and virulence of identified pathogen was determined using Koch's pathogenicity postulates. For this, sterilized petri plates were taken lined with 2 filter papers moistened with 2 mL of double distilled water. The detached leaves from healthy plants were placed in petri plates in such a way that their petiole ends touched the moisten filter papers. Then approximately 2 mL ($5\times10^5$ spores/mL) of spore suspension was inoculated on the leaf surface and plates were incubated at 25±2 °C and observed regularly for the emergence of disease symptoms. After the onset of disease, re-isolation of pathogen from the infected leaves was carried out to fulfil the Koch's pathogenicity postulates. Analysis of disease progress over time in detached leaf method revealed irregular but sometimes concentric brownish spots on leaf laminae that were caused by pathogenic spread on rose leaves. These brown spots turned to reddish or blackish in color after 10-15 days and about 95% of the leaf area was found to be infected (Figure 4).

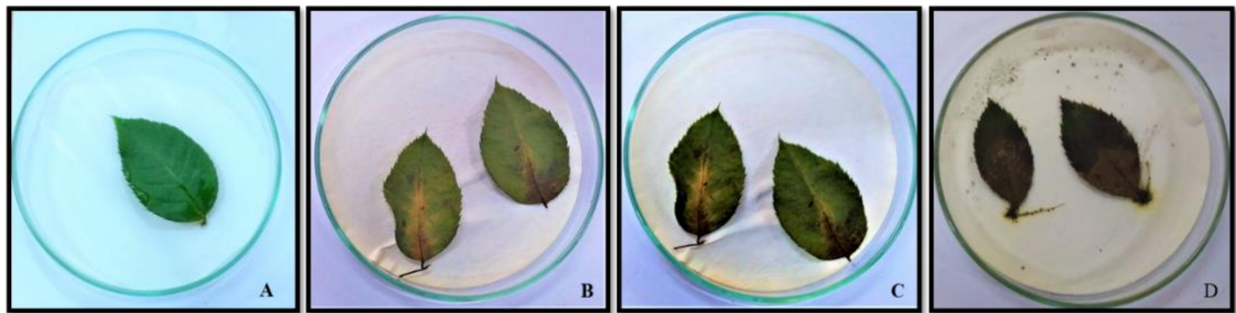


Figure 4. Disease symptoms of *Rosa indica* caused by *Alternaria tenuissima*.

Further, pathogenicity test was conducted in pot trials to confirm the efficacy level of isolated pathogen by spraying on one month old *R. indica* plantings. For this, 8 days old fully grown culture of pathogen was used as spore suspension (2 mL) that contained $5\times10^5$ spores $mL^{-1}$. After inoculation, infectious symptoms were observed within 15 days. Symptoms that appeared first were developed on young leaves as yellow round or irregular spots, which became brown as the disease progressed with sizes ranging from small spots to approximately 1.2 cm in diameter. Some spots coalesced to form larger lesions. Infected area of *R. indica* plants was recorded for the evaluation of disease severity caused by the pathogen. The virulent aptitude was demonstrated by *A. tenuissima* as about 92% disease severity was noticed by pathogen (Figure 5).

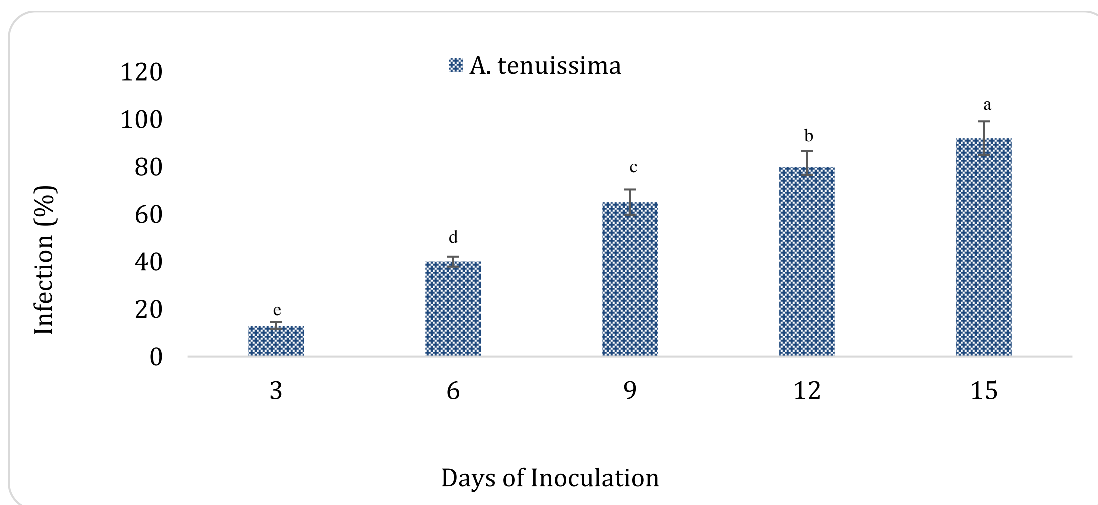


Figure 5. Analysis of disease severity by *Alternaria tenuissima* on *Rosa indica*.
Vertical bars indicate standard errors of means of three replicates. Values with different letters show significant difference by ANOVA as determined by statistix 8.1 software, LSD test at p≤0.05.

The study concludes the novel representation of *A. tenuissima* as a leaf spot pathogen of *R. indica*. It will assist to recognize the actual biological agent causing damage to crop and will help to control the pathogen in broad spectrum rather than use of fungicides and chemicals.

## ACKNOWLEDGEMENTS

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**Contribution of Authors:**
All authors contributed equally to this work.