

Occurrence of *Alternaria alternata* causing Alternaria blight in pigeonpea in India

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ABSTRACT

Blight symptoms on pigeonpea were observed in alarming proportion since 2009 onwards in Andhra Pradesh state of India. *Alternaria* blight infected pigeonpea plants were collected from Andhra Pradesh state of India to isolate and characterize the pathogen. The isolate proved pathogenic on pigeonpea cultivar ICPL 87119. Genetic characteristics were analyzed based on the sequence of the rDNA-internal transcribed spacer (ITS) region. The phylogenetic tree based on rDNA-ITS analysis showed that the *Alternaria alternata* causing Alternaria blight in pigeonpea is very distinct from the other *Alternaria* isolate reported from different host. This is the first report of molecular identification of *Alternaria alternata* causing Alternaria blight in pigeonpea.

Keywords: Climate Change; Internal Transcribed Spacer (ITS); PCR; Pigeonpea; Phylogenetic Tree

1. INTRODUCTION

Pigeonpea [*Cajanus cajan* (L.) Millsp.] is the most versatile grain legume crop grown in the semi-arid tropical and subtropical regions in Asia, Africa, and America [1]. Globally, pigeonpea is cultivated in about 4.5 million ha, adding 3.48 million tonnes of grain to global food production [2]. India is a major pigeonpea producer in the world with a contribution of 75 - 80 percent. Pigeonpea is a major source of protein; enriches soil; provides fodder and fuel wood; and it is beneficial for arresting soil erosion [3,4]. Pigeonpea crop has a direct bearing on the economic and financial well-being and on the nutritional status of the subsistence farmers in the subcontinent as it is a low-input, rainfed crop and provides economic returns from each and every part of the plant. However,

average yields of pigeonpea are low (450 - 670 kg per ha) and diseases are the major constraints to the high yield potential of pigeonpea cultivars. Pigeonpea is susceptible to many diseases [5]. Recently due to the changes in climate, blight symptoms on pigeonpea were observed in alarming proportion in major chickpea growing regions particularly in Andhra Pradesh and Karnataka from last 4 - 5 years. The disease was observed in 60% of the fields surveyed and incidence ranged from 20% - 80% during irrespective of cultivars sown. Infected plants in the field showed symptoms on all aerial parts of the plant (leaves, stems, buds, and pods). Symptoms on leaves were small, circular, necrotic spots that developed quickly forming typical concentric rings [6]. Later, these spots coalesced and caused blighting of leaves. Spots were initially light brown and later turned dark brown. On stems, spots were sunken with concentric rings. In severe infection, defoliation and drying of infected leaves, branches, and flower buds were observed (**Figure 1**).

Accurate identification of the pathogen is the preliminary step for further R&D on the biology of the pathogen and epidemiology of disease. Different molecular methods such as RAPD, RFLPs, DNA hybridization, AFLP, and DNA sequences [7-11] have been used to reveal the molecular identification as well as genetic polymorphism. No such reports regarding molecular identification of the causal organism of Alternaria blight of pigeonpea have been described yet.

Present study reports the frequent occurrence of Alternaria blight in pigeonpea and its emerging threat to pigeonpea production.

2. MATERIALS AND METHODS

2.1. Fungal Isolate Origin

Blight infected pigeonpea plants were collected from Andhra Pradesh state of India. Isolation was made by plating surface sterilized (0.8% sodium hypochlorite for

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2 min) pieces from blight infected pigeonpea plant parts (leaves, stem, bud etc.) on potato dextrose agar (PDA) medium. Inoculated plates were incubated at $25^{\circ}\text{C} \pm 2^{\circ}\text{C}$ for colony growth. The cultures were purified by single spore isolation. The culture was tentatively identified as *Alternaria* spp. based on standard mycological parameters and maintained on PDA slant at 4°C for further study.

2.2. Pathogenicity Test

To confirm pathogenicity, 8 - 10 days old seedlings of pigeonpea cultivar ICPL 87119 were grown in pots in three replications (5 plants/pots). Conidial suspension of *Alternaria* sp. was prepared from 7 days old culture grown on potato dextrose broth (PDB). Seedlings were spray inoculated with conidial (5×10^5 conidia/ml) suspension and covered with polythene covers and incubated at $28^{\circ}\text{C} \pm 1^{\circ}\text{C}$ and 12 h photo period. Un-inoculated pots served as control. Polythene covers were removed after 48 hrs. Plants were regularly watered and monitored for disease development.

2.3. DNA Extraction

For DNA extraction, the pathogen was grown on PDB medium at $25^{\circ}\text{C} \pm 2^{\circ}\text{C}$ for 8 days. Mycelia were harvested by filtering through Whatman filter Paper No. 1, washed repeatedly with distilled sterilized water to remove excess salts adhering to it. DNA extraction was based on the cetrimide tetradecyl trimethyl ammonium bromide (CTAB) method [12]. Mycelium (1 g) was crushed in liquid nitrogen and transferred into 7.5 ml pre-warmed (65°C) DNA extraction buffer [1 M Tris-HCl (pH 8.0), 5 M NaCl, 0.5 M EDTA (pH 8.0) and 2% CTAB], mixed well and incubated in a water bath at 65°C with gentle shaking for 45 min. Equal volume of chloroform: isoamyl alcohol (24:1 v/v) was added and mixed gently to denature proteins and centrifuged at 12,857 g for 10 min. DNA was precipitated with 0.6 volume of chilled ethanol and 0.1 volume of 3 M sodium acetate (pH 5.2) and centrifuged at 18,514 g for 15 min. The pellets were washed twice with chilled 70% ethanol, dried at room temperature, re-suspended in 100 μl sterile TE (10 mM Tris-HCl buffer and 1 mM EDTA—pH 8) and stored at -20°C deep freezer. Isolated DNA was electrophoresed in 1.0% agarose gels to check the quality and concentration.

2.4. Sequencing of rDNA-ITS Region

The pathogen *Alternaria* sp. identified tentatively on morphological level was characterized at molecular level using rDNA-ITS region sequencing. ITS 1 and ITS 4 primer pair were used for PCR amplification of the pathogen [13]. The PCR reaction contained 50 ng genomic

DNA, 1X PCR buffer (Thermo scientific), 1.5 mM MgCl_2 , 0.2 mM dNTPs, 0.25 μM of each primer and 1.0 unit Dream *Taq* DNA Polymerase (Thermo scientific) in a 50- μL reaction volume. The PCR program was: one cycle at 94°C for 2 min, 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s, and one cycle of 72°C for 5 min, and then held at 4°C . PCR amplicon was purified with a Wizard[®] SV Gel and PCR Clean up system (Promega, USA) according to the manufacturer protocol and then sequenced from commercial service (Xcelris Labs Limited, Ahmedabad).

2.5. Genetic Data Analyses

Sequence identity matrix of *Alternaria* sp. was generated using Bioedit Sequence Alignment Editor (version 5.0.9) [14]. After multiple alignment, phylogenetic analysis was done in MEGA 4.0 software [15] using the default parameters of one character-based algorithm. The bootstrapped consensus phylogenetic tree was generated for each of these algorithms with 500 replication.

3. RESULTS AND DISCUSSION

3.1. Fungal Isolate

The fungus produced abundant, branched, septate, brownish mycelia. Conidiophores were simple, olive-brown, septate, variable in length with terminal conidia, which were solitary or in short chains. Mature conidia measures from $10 - 30 \times 5 - 12 \mu\text{m}$, short conical beak or beakless, narrowly ellipsoid to ovoid and elongated on branching chains. Juveniles were narrowly elliptical, 3 - 7 transepta, 1 - 5 longisepta and have individual chains of 5 - 15 conidia. Conidial characteristics from culture were similar to the conidia isolated from infected plants. Based on the morphological characters (Figures 1 and 2), the organism was identified as *Alternaria alternata*.

3.2. Pathogenicity Test

The pathogen proved pathogenic on pigeonpea cultivar ICPL 87119 and identical disease symptoms as observed



Figure 1. (A) *Alternaria* blight symptom on leaves and (B) aerial plant parts (leaves, stem, buds and pods).



Figure 2. Conidial and mycelial morphology of *Alternaria alternata*.

in the field symptoms on leaves were small, circular, necrotic spots that developed quickly forming typical concentric rings. Later, these spots coalesced and caused blighting of leaves. Spots were initially light brown and later turned dark brown were observed 10 days after inoculation. No symptoms were observed on control plants.

3.3. Sequencing of rDNA-ITS Region

To confirm the molecular identity of *A. alternata*, the rDNA-ITS region of the present isolate was amplified and sequenced. The rDNA sequence was deposited in the GenBank database under the accession no JQ074093. The size of the PCR product was 490 bp. BLAST analysis of the 490-bp amplicon showed 100% similarity with *A. alternata* sequences (JN542524) reported from other host from India. To assess the relationships with other *Alternaria* spp. the corresponding genomic regions of 17 *Alternaria* sp. showing 99% - 100% sequence identity with present isolate were obtained from GenBank and a phylogenetic tree was constructed. Phylogenetic analysis revealed that the present *Alternaria alternata* infecting pigeonpea grouped separately from other *Alternaria* spp. reported from other host spp. (**Figure 3**).

The rDNA-ITS analyses performed on genomic DNA of *A. alternata* isolate revealed the presence of high level of genetic diversity with other *Alternaria* isolates infecting other host. The rDNA-ITS has the unique potential for providing information across an entire genome. The isolates showed strong genetic similarity within a range of 99% to 100%, indicating high level of identity among them irrespective of host. In phylogenetic tree, the present isolate grouped separately from the other isolates from different host indicating *A. alternata* infecting pigeonpea is distinct from other *Alternaria* spp. reported from different host.

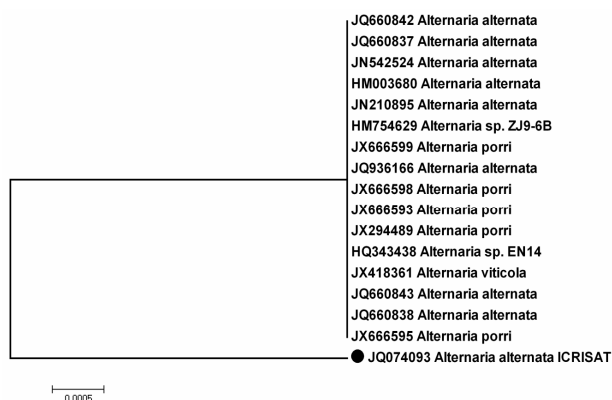


Figure 3. Phylogeny tree showing relationships among the related *Alternaria* spp. with *Alternaria alternata* (●) based on their ITS sequences.

4. CONCLUSION

Alternaria alternata causing *Alternaria* blight in pigeonpea was characterized first time at molecular level using rDNA-ITS region. rDNA-ITS analysis indicated that *Alternaria alternata* causing *Alternaria* blight in pigeonpea is a distinct species from other reported *Alternaria* spp. infecting different host. In our earlier findings, we [16] have reported *Alternaria tenuissima* causing *Alternaria* blight in pigeonpea.

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