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Molecular phylogenetic analysis of *Alternaria alternata,* a new pathogen associated with postbloom fruit drop of *Citrus reticulata*

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Postbloom fruit drop of *Citrus reticulata* was noticed in fields during early development of fruits. Disease symptoms appeared as round brownish black spots, lesions are usually surrounded by yellow aureole. Black lesion can be seen after peeling the fruit, thereby reducing the value of fruit for the fresh market. Fungal pathogen isolated from infected fruit was identified as *Alternaria alternata* disease symptom by the isolated pathogen was established in healthy mandarin fruit following completion of Koch's postulate. Morphological and molecular identification confirmed the pathogen as *Alternaria alternata* , which is the first report of postbloom fruit drop of *Citrus reticulata* in India. Genomic DNA was prepared from *A. alternata*, purified and PCR amplification of 18S rDNA was done using ITS region specific primer pair. The product size was approximately 544 bp. Phylogenetic tree based on the neighbor-joining analysis of gene sequences of *A. alternata* from citrus (IPL.CIT.A.F1.001) showed satisfactory homology with 16 ex type strains of *Alternaria alternata* sequences from NCBI GenBank data base.

Key words : *Alternaria alternata, Citrus reticulata*, fruit drop, mandarin

INTRODUCTION

Indian horticulture is the core sector of agriculture, representing a broad spectrum of crops and production of a wide range of horticultural commodities. *Citrus reticulata* Blanco belongs to the family Rutaceae is one of the major economic horticultural crops in India. It comes after the production of Mango and Banana in India (Das *et al*. 2007). An assessment for conservation and utilization of genetic diversity of Citrus in India has been reported by Malik *et al.* (2006). In North Bengal especially in the Darjeeling hills a large number of growers are involved in mandarin cultivation. Annual production of citrus in India is six million metric tons and that of Darjeeling hills is 67030 fruits/ha. Production and productivity of any cash crop is directly dependent on its health status which is correspondingly related to pest and disease management along with its nutritional input.

Darjeeling mandarin is very commonly subjected to the attack of many pest and fungal pathogens, such as *Fusarium solani* (Allay and Chakraborty, 2010) *Macrophomina phaseolina* (Chakraborty *et al*. 2012) causing root rot disease. Root colonization of Citrus plants with arbuscular mycorrhizal fungi (AMF) have been documented (Allay *et al*. 2021). Recently immunodetection of dominant AM fungi (*Rhizophagus fasciculatus* and *Gigaspora gigantea*) in soil and root tissues in mandarin plants, their exploitation as bioinoculants and cellular localization of defense enzymes following induced immunity developed against *Fusarium solani* have been elucidated (Chakraborty and Allay, 2022).

Intense infection on the fruit samples of *Citrus reticulata* (Darjeeling mandarin), grown in the experimental field (26p 42'22''N 88p 20'55''E) of University of North Bengal. was observed during August-October. The fruit drop was noticed in field during early growth of fruits. In the present communication we have identified the causal organism, *Alternaria alternata* which is the first report of this fungal pathogen causing postbloom fruit drop on *Citrus reticulata* in India.

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MATERIALS AND METHODS

Plant sample

The mandarin plants (Fig 1,A) were grown in the experimental field under ideal conditions - average maximum temperature of 31°C and minimum temperature of 21°C, the average rainfall 201 mm and humidity (65–78%). Plants were not subjected to any other physiological conditions or pathogens.

Pathogen isolation

In the month of October the infected fruit samples were collected in plastic bags from the experimental field (26^0 42′22′′N 88^0 20′55′′E) and quickly transferred to the laboratory. Collected fruits were washed with tap water properly. Infected region was cut into 0.5 cm^2 pieces and sterilized with 0.1% $HgCl_2$ for 30-50 seconds. Small pieces of infected regions were soaked three times in sterile distilled water for 40-60 seconds, dried in paper towel and transferred onto Petri dishes containing potato dextrose agar (PDA) supplemented with streptomycin sulphate and incubated for 7 days at 27º C to elevate growth and sporulation. Microscopic observations were made for the isolated fungal pathogen. The morphological identification was done by comparing the mycelium behavior, sporulation, spore structure, germination pattern, branching patterns, conidial size and applying identification key as described by Simmons (2007).

Pathogenicity test

Fresh mandarin fruits were obtained from the field, washed thoroughly with sterile distilled water and surface sterilized with 70% ethanol. Conidial suspension (5×10^4 conidia/ml) prepared from 7 day-old-sporulated culture of *A. alternata* and 100 µl was inoculated by the sterile Dispo Van syringe in freshly collected mandarin fruit. Sterile distilled water was inoculated in the control set. Inoculated fruits were incubated at 27º C for 10 days. Experiments were repeated twice and re-isolation of pathogen from infected fruits were made in order to complete the Koch's postulates. .

Preparartion of genomic DNA

Fungal mycelia (5 day old) of *A. alternata* grown in potato dextrose broth (PDB) was crushed with liquid nitrogen and incubated with lysis buffer containing 250 mM Tris-HCl (pH 8.0), 50 mM EDTA (pH 8.0), 100 mM NaCl and 2% SDS, for 1 h at 65^0C followed by centrifugation at 12,000 rpm for 15 min. The supernatant was extracted with equal volume of water saturated phenol, centrifuged at 12,000 rpm for 15 min and further extracted with equal volume of phenol : choloroform : isoamyl alcohol (25:24:1) by centrifugation at 12,000 rpm for 15 min; the aqueous phase was transferred in a fresh tube and choloroform (1:4 v/v) was added followed by 0.5 M Na-acetate (1:10 v/v). Next isopropanol was added to the above mixture and centrifuged. DNA was precipitated from the aqueous phase with chilled ethanol (100%) and pelleted by centrifuging at 12,000 rpm for 15 min followed by washing in 70% ethanol and centrifugation. The pellets were air dried and suspended in Tris-EDTA (TE-1X) buffer (pH 8.0).

PCR amplification

Genomic DNA was amplified by mixing the template DNA, with the polymerase reaction buffer, dNTP mix, primers (ITS1 and ITS4) and Taq polymerase. Polymerase chain reaction (PCR) was performed in a total volume of 100 µl, containing 78 µl deionized water, 10 µl 10X Taq pol buffer, 1 µl of 1 U Taq polymerase enzyme, 6 µl 2 mM dNTPs. 1.5 µl of 100 mM reverse and forward primers and 1 µl of 50 ng template DNA. PCR was programmed with an initial denaturing at 94^0C for 5 min followed by 30 cycles of denaturation at 94^0C for 30 s, annealing at 59^0C for 30 s and extension at 70^0C for 2 min and the final extension at 72^0C for 7 min in a Primus 96 advanced gradient Thermocycler. PCR product (20 µl) was mixed with loading buffer (8 µl) containing 0.25% bromophenol blue, 40% w/v sucrose in warer, and then loaded in 1% agarose gel with 0.1% ethidium bromide for horizontal electrophoresis and then visualized under UV. Concentration of the amplicon was checked in a Nanodrop ND 2000.

Sequencing and phylogenetic analysis

The amplicon was then purified using PureLink purification column (In vitrogen). Sequencing of amplicon with forward and reverse primers carried out in ABI 3730xl cycle sequencer. Forward and reverse sequences were assembled and contig was generated after trimming the low quality bases. The sequence analysis was carried out using

bioinformatic tool BLAST of NCBI. OPA10-2 sequences were aligned using Mega 4.0 software. Support for clades was estimated using non-parametirc bootstrapping with 100 pseudo-replicated datasets in Mega. Consensus tree was produced by Mega 4.0.

RESULTS

Disease symptoms

The fruit drop was noticed in fields during early development of fruits. Disease symptoms appeared as round brownish black spots, lesions are usually surrounded by yellow aureole and size of the lesion varies from (0.3-5.1cm) in diameter (Fig.1, B-C). Black lesion can be seen after peeling the fruit (Fig 1D). On fruit, lesions vary from small dark necrotic spots to large sunken pockmarks, thereby reducing the value of fruit for the fresh market.

Pathogen identification

Identification of the fungal pathogen was done following microscopical observation of spore characteristics. The fungal mycelia are simple or branched, initially white in colour and later it alter into olive green, and then to grey black. Conidia (Fig 1 E) are smooth, slightly elongated apical cell, ovoid, obclavate, large, dark coloured, muticellular with longitudinal and transverse septation. It generally shows 3-8 transverse and 0-3 longitudinal septa and average size measured to (22 to 38 μm) × (5.8 to 11.2 μm). Conidia shows yellowish to golden brown in colour. Conidium germinated quickly at high humidity and nutrient free sterile distilled water its germ-tube grew to form extensive hyphal network (Fig 1F). Based on the morphological characters (Fig 1E-F) fungal pathogen was identified as *Alternaria* sp.

Completion of Koch's postulate

Artificially inoculating fruits of citrus show initial development of small dark lesion demonstrate the initiation of disease. Disease symptoms developed within 5 to 7 days on inoculated fruits (Fig 2A) and control fruits were not affected. This study revealed that the isolated fungus was pathogenic to citrus. Re-isolation of fungal pathogen confirmed the identity of the causal organism as *Alternaria* sp. following microscopic observation of conidia (Fig 2B) and germination of conidia (Fig 2C). Disease severity was measured according to 0-3 scales as described by Peever *et al.* (2005).

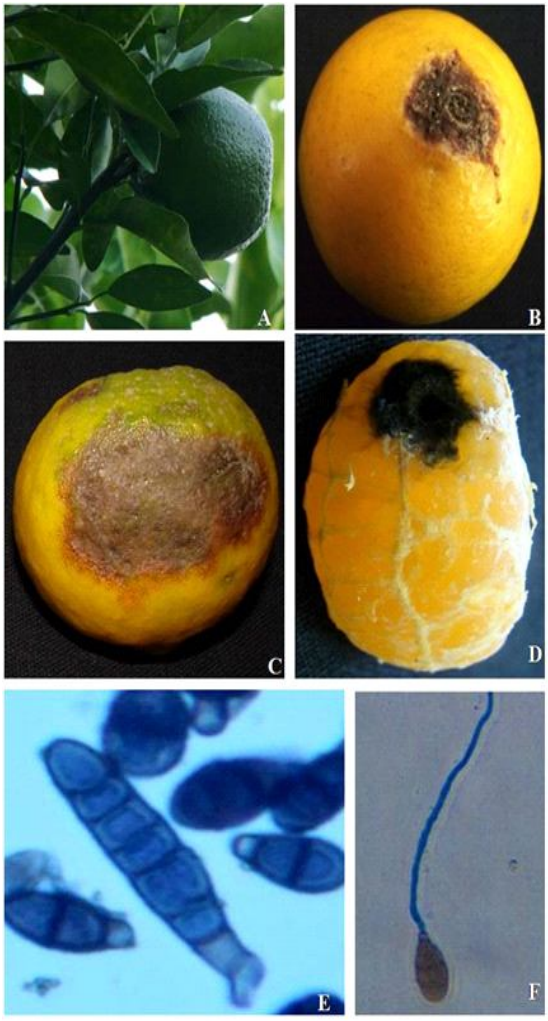

Fig.1. (A) Healthy mandarin plant (*Citrus reticulata*) growing in experimental field, (B) Postbloom mandarin fruit drop showing symptom, (C) Disease progression (D) Symptoms showing after peeling the fruit (E) Conidia of *Alternaria alternata* (F) Germinated conidium

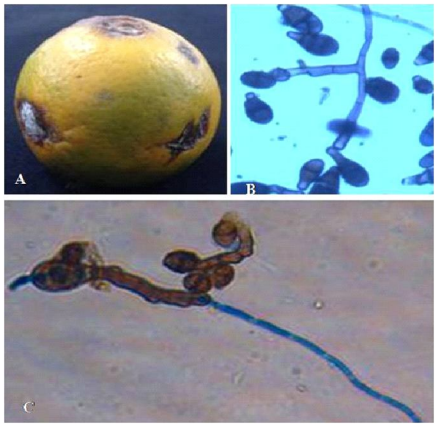

Fig.2 (A) Mandarin fruit showing symptom following artificially inoculation with *Alternaria alternata*, (B) Hyphae and conidia of reisolated fungal pathogen (*A. alternata*), (C) Germinated conidium

Table 1. The nucleotide sequence used for ITS PCR

Seq name	Primer seq 5' – 3'	Mer	TM	% GC	Amplicon size (bp)
ITS 1	TCCGTAGGTGAACCTGCG	18	61	56	544
ITS 4	TCCTCCGCTTATTTGATATGC	21	63	59	

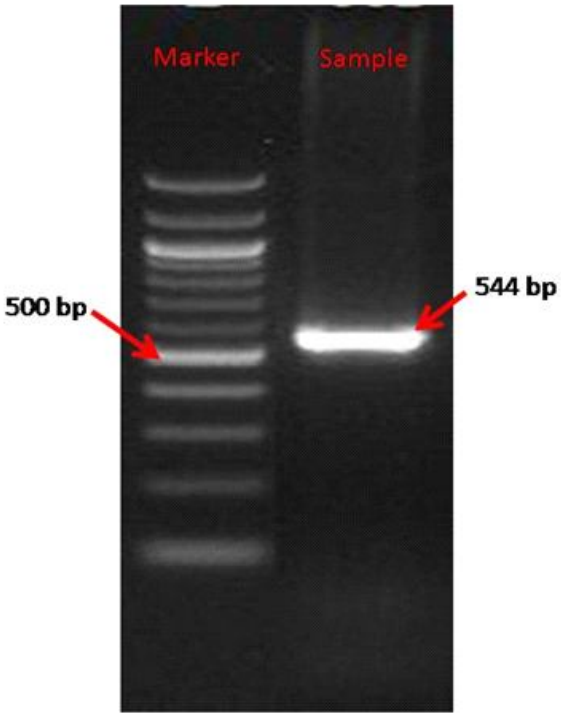

Fig. 3. PCR product of amplified DNA of *Alternaria alternata*

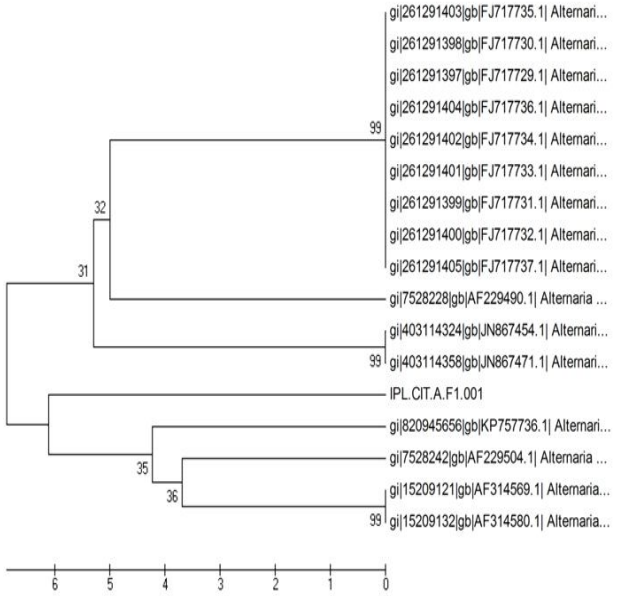

Fig. 4. Phylogenetic tree based on the neighbor-joining analysis of gene sequences of *Alternaria alternata* from citrus (IPL.CIT.A.F1.001) and of other citrus associated *Alternaria* species deposited in GenBank (isolate number and GenBank accession number provided in the figure). The significance of each branch is indicated by a bootstrap percentage calculated for 1,000 subsets.

ITS – PCR and phylogenetic analysis

PCR products of genomic DNA of the isolated fungal pathogen using nucleotide sequence used for ITS PCR has been presented in Table 1 which revealed amplicon size of 544 bp (Fig 3).

The sequence analysis disclose 100% similarity between the ITS region of *Alternaria alternata* in GenBank. Phylogenetic analysis disclose that it consist 5 clades. Phylogenetic analysis were inspected by MEGA package (version 4.01; Institute of molecular Evolutionary Genetics, University Park, PA) as described by Tamura *et al* (2007). Phylogenetic conclusion was established by the UPGMA method. *Alternaria alternata* sequence shows satisfactory homology with 16 ex type strains of *Alternaria alternata* sequences from the NCBI Genbank data base (Fig.4) as analysed by BLAST.

DISCUSSION

The issue of climate change and climate variability has thrown up greater uncertainties and risks further imposing constraints on mandarin production systems. The challenges ahead are to have sustainability and competitiveness to achieve the targeted production to meet the growing demands in the environment of declining land, water and threat of climate change which needs innovation and its adoption for improving production in challenged environment. This is the first report of a pathogenic *Alternaria alternata* causing postbloom fruit drop of *Citrus reticulata* in India. The key described by Simmons (2007) for *Alternaria sp*. isolates were analysed and the morphological characters are alike to those of *Alternaria alternata*. Molecular, ecological and evolutionary approaches have been made by

Akimitsu *et al* (2003), in order to understand fruit drop disease of citrus caused by *Alternaria* species, which also causes other diseases of citrus including, brown spot of tangerine, leaf and fruit spot of rough lemon, Rangpur lime and Mexican lime in semi-arid and humid condition (Peever *et al*.2005). The lesions are observed on the leaves, twigs and immature fruit of citrus (Peever *et al*. 2002; Timmer *et al.* 2003) and cause decreased productivity and complete loss of commercial value of infected fruits. Molecular phylogenetic studies suggest that the isolates of *Alternaria* from citrus plants should be designated as *Alternaria alternata* (Peever et al., 2004). Bella *et.al.,* (2011) have also characterized *Alternaria alternata* isolates from Tangerine hybrids affected by Brown spot in Italy. Two pathotypes of *Alternaria alternata* are currently known based on the production of host-specific toxins (HST): ACT— toxin of the tangerine pathotype, which is specific to tangerine (*Citrus reticulata* Blanco), and their hybrids; and ART— toxin of the lemon pathotype, which affects rough lemon (*C. jambhiri* Lush), and Rangpur lemon (*Citrus limonia* Osbeck). Postbloom fruit drop (PDF) of citrus, restricted to flowers of sweet orange and most other citrus, whereas key lime anthracnose (KLA), a disease of foliage, flowers and fruits of key lime only have also been reported to be caused by distinct phylogenetic lineages of *Colletotrichum acutatum* (Peres *et al*., 2008)

In this study *Alternaria alternata* was identified as the cause of postbloom fruit drop of manadarin in Darjeeling hills. The ribosomal RNA genes (rDNA) own characters that are capable for recognition of fungus at species level. These rDNA are firm and display a preserve and various regions with in the genome. Internal transcribed spacer (ITS) regions have been used profitably to initiate particular primers capable of discriminate closely related fungal species. The ITS1 sequences of *Alternaria alternata* and *Alternaria tenuissima* are 99-100% identical. So these two species cannot be recognizable by ITS1 sequence. As reported by Pryor *et al* (2002), two genomic region tubulin sequence and ITS sequence are to be used for fungal systematic. The phylogenetic analysis revealed that the accession number KP757736.1 is closely related with AF229504.1. Besides, accession number AF314569 and AF314580 are closely related and placed under same clades. Molecular phylogenetic relationships amongst *Alternaria* species and related fungi based upon analysis of nuclear ITS and mtSSU rDNA sequences have been described (Pryor *et al*. 2000). Peever *et al.* (2005) reported that the sequenced endopolygalacturonase gene (endoPG) and two region of genomes (OPA1-3 and OPA2-1) of *A. alternata* isolates which were taken out from brown spot lesions showed genetic variance. Phylogenetic analysis done by Masha *et al.* (2014) have also disclosed the genetic differences in *Alternaria alternata* isolates associated with brown spot in tangerine cultivars.

Recognition of genetic diversity has an important role in the epidemiological studies and disease management of *Alternaria alternata* of citrus. A web-based postbloom fruit drop disease alert system has been discussed by Perondi *et.al*. (2000) as citrus advisory system, which highlights (a) decision support system to monitor and predict the risk of postbloom fruit drop on citrus, (b) reducing the fungicide application following the recommendations of the system and (c) using leaf wetness and temperature to predict disease outbreaks on citrus. All the factors are necessary to develop a disease forecasting models and the assessments of genetic and pathogenic diversity are to be considered for a perfect management of postbloom fruit drop of citrus.

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