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Alternaria blight is one of the most importatnt fungal disease of sunflower caused by *Alternaria helianthi* (Hansf.) Tubaki and Nishihara. However, mixed infection of more than one *Alternaria* species has been reported from China, North korea and Pakistan.The objective of this study was to characterize the specie associated with the leaf blight of sunflower. *Alternaria* isolates were isolated from sunflower leaves showing typical dark brown to black, circular to irregular spots during *kharif*, *rabi* and summer at ZARS, GKVK, UAS-Banglore. The species obtained were morphologically categorised as*A. helianthi* and *A. Alternate* based on spore morphology and cultural characteristics. Through molecular characterization the same species were confirmed as *A. heliatnhi* and *A. alternata* based on ITS rDNA sequencing using universal primers ITS1 and ITS 4 and the sequences were deposited in NCBI with GenBank accession numbers.

Key words: *Alternaria* spp., sunflower, blight, characterization

INTRODUCTION

Sunflower is an important oilseed crop in India, occupies the fourth place among oilseed crops in terms of acreage and production. The area under sunflower cultivation in India was 0.59 m ha, with a total production of 0.43 mt and productivity of 736 kg/ha. Commercially available sunflower seeds contains 35 to 45 per cent oil in the seed. It is primarily grown for edible oil characterized by a high concentration of linoleic acid and moderate level of oleic acid. Among the biotic stresses the diseases are one of the major constraints in successful sunflower production. In Karnataka, the major diseases of sunflower are necrosis virus, *Alternaria* blight, powdery mildew, rust, collar rot and downy mildew. Among these *Alternaria* blight has been considered as a potentially destructive disease in many parts of sunflower growing countries (Allen *et al*. 1983) and in Karnataka (Shankergoud *et al*. 2006). The disease has been known to cause reduction in flower size, number of seeds per head, seed yield per plant, seed weight and also oil content. The loss in yield varied from 11.30 to 73.33 per cent depending on the extent of infection (Balasubramanyam and Kolte, 1980). In Northern Karnataka, *Alternaria* leaf spot is known to cause more than 80 per cent of the yield loss under epiphytotic conditions (Hiremath *et al*. 1990; Amaresh, 1997). The nature of yield reduction is determined by the stage of plant growth when disease epidemic develops Allen *et al.* (1981). More than one *Alternaria* species of have been found to cause leaf blight on sunflower. The present study aims at identifying the *Alternaria* spp. causing sunflower blight.

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MATERIALS AND METHODS

Isolation, purification and identification of pathogen

The sunflower leaves showing typical dark brown to black, circularto irregular spots were collected from the field in *kharif* (wet)*, rabi* (dry) and summer 2016 at Zonal Agricultural Research Station, GKVK, UAS-Banglore and brought to the laboratory for isolation of the causal agent.

Leaf blight infected specimen was microscopically examined for confirmation of the fungus. After confirmation of the fungus as *Alternaria*, infected

leaves exhibiting typical *Alternaria* leaf blight symptoms were selected and pathogen was isolated by following standard tissue isolation method. The infected leaf bits measuring about2 mm were disinfected with 1% NaOCl solutions for a minute and rinsed thrice with sterilized water to remove traces of disinfectant. These pieces were then transferred to petri plates containing Potato Dextrose Agar (3 pieces/dish). These plates wereincubated at 27 ± 1°C. After 10 days of incubation thegrowth of the fungus in association with the leaf spot was observed. Following hyphal-tip technique, test pathogen was transferred aseptically on the PDA slant in test tubes. Single spore isolation technique was followed for the purification of pathogen. The obtained species were initially identified as *A. alternata* and *A. helianthi* based on spore morphology and colony character.

Pathogenicity test

Ten days old culture of both species were used for proving the pathogenicity by applying Koch's postulates. For this purpose, surface sterilized seeds of sunflower hybrid KBSH-44 which is susceptible to *Alternaria* blight were sown in the earthen pots filled with autoclaved potting mixture of Soil:Sand:FYM (2:1:1). Healthy growing sunflower seedlings were maintained, watered regularly and kept in the glass house for further studies. Conidial suspension (10^6 conidia /ml) of 10 days old test fungus were inoculated to 30 days old seedlings. Uninoculated seedlings of the same age sprayed with sterilized water served as control. After inoculation, the seedlings pots (both inoculated and uninoculated) were incubated in the glass house, where relative humidity (80 to 90%) and optimum temperature (27±1°C) were maintained for development of *Alternaria* blight symptoms.

Morphological characterization

Cultural studies

Colony characters. Colony growth pattern of 20 day old cultures of both the species, grown on PDA at 27 ± 1 ^{0}C temperature were recorded.

Molecular characterization

DNA extraction by CTAB method

The mycelium of both the species collected from the liquid cultures in potato dextrose broth after 5 days of incubation was filtered through Whatmans No-40 filter paper. The mycelia were then dried completely by pressing in between folds of pre-autoclaved filter papers. The DNA extraction method was standardized and certain steps were optimized to produce good concentration of DNA using plant DNA isolation kit (CTAB method). Mycelium of 0.5 g was taken and ground in a Pestle and Mortar with nine ml of CTAB extraction buffer, mixed gently by inversion. For 60-90 minutes the tubes were incubated at 65 ^{0}C, with occasional inversion. The samples were allowed to cool by keeping the tubes in a trough of water at room temperature.

Five ml of chloroform: isoamyl alcohol (24:1) was added, the tubes were gently to mix the content for five minutes. The samples were subjected to spinning in a centrifuge for 15 minutes at 6500 rpm at room temperature. The aqueous layer was transferred to a fresh tube and 25 RNase A (20 mg ml^{-1}) was added. The samples were mixed gently by inversion and incubated for 30 min at room temperature. Six ml isopropanol was added to each tube and mixed gently by inversion until a white fluffy DNA precipitate appeared. The contents were centrifuged at 6500 rpm for 15 min to pellet the DNA. After 2-3 min, eight ml of cold wash buffer was added and incubated for 20 min at room temperature. The tubes were centrifuged to pellet the DNA at 6500 rpm for 15 min. The supernatant was discarded and eight ml of cold 70 per cent ethanol was added to the tube containing the DNA pellet. One ml of elution buffer was added and mixed gently to dissolve the pellet and kept at 4 ^{0}C overnight. The DNA solution appeared to be turbid after standing overnight at 4 ^{0}C and the samples were heated to 65 ^{0}C for centrifugation at 6500 rpm for 15 min and the clear supernatant containing DNA was transferred to a fresh 1.5 ml tube discarding the pellet.

Qualitative and quantitative verification of DNA

The quality and quantity of DNA was analyzed by running 2 µl of each sample mixed with 2 µl of 10x loading dye in 1% agarose gel. The DNA from all the isolates produced clear sharp bands in one per cent agarose gel indicating good quality of the DNA. The DNA was quantified by comparing with the 1 kb size marker (Genei Pvt. Ltd. Bengaluru). The gel was observed under UV light and docu-

mented using gel documentation unit.

PCR amplication of ITS region

The ribosomal DNA (rDNA) unit contains genetic and non-genetic or spacer region. Each repeat unit consists of a copy of l8S, 5.8S and 28S like rDNA and its like Internal Transcribed Spacers (ITS) and Inter-Genic Spacers (IGS). The rDNA have been employed to analyze evolutionary events because it is highly conserve, whereas ITS rDNA is more variable. Hence, it has been used for investigating the species level relationships.

The primers for amplification were custom synthesized at Bengaluru Genie Pvt. Ltd. and supplied as lyophilized products of desalted oligos. Primer sequences used were as follows

Organism	Sequence	
Fungal ITS	ITS-1-F	5´-ACC GTT ACC AAA CTG TTG -3´
	ITS-4-R	5´-AAG TTC AGC GGG TAT CCT -3´

PCR reaction mixture

Reactionmixture	Quantity
Template DNA	1.5 µl
Primers F and R	1 µl
dNTP´s (2 Mm)	0.8 µl
Taq buffer A (10X)	2 µl
Taq DNA polymerase (3U/ µl)	0.2 µl
$MgCl_2$	1 µl
Sterile Water	3.5 µl
Total	10 µl

PCR condition for ITS region amplification

Step	Temp (^{0}C)	Duration (min)
Initial denaturation	94	5
Denaturation	94	1
Annealing	54	1
Extension	72	2
Final extension	72	10
Hold	4	20
No. of cycles Denaturation Annealing Extension	30	

Separation of amplified products by agarose gel electrophoresis

Agarose gel electrophoresis was performed to resolve the amplified product using 1.4 per cent agarose in IX T BE (Tris Borate EDTA) buffer, 0.5 ug ml^{-1} of using ethidium bromide and loading buffer (0.25 % Bromophenol Blue in 40 % sucrose). Four µl of the loading dye was added to 20 µl of PCR product and loaded to the agarose gel. Electrophoresis was carried at 65 V for 1.5 h. The gel was observed under UV light and documented using gel documentation unit.

Sequencing of ITS region

The ITS region was sequenced for 2 speciesbelonging to same Agro-climatic conditions to confirm organism.

3.5.8 Sequencing and in silico analysis

The PCR product was sequenced using forward and reverse primers at Sakhala enterprise Bengaluru. Homology search done using BLAST algorithm available at the VA3T.ncbi.nlm.nih.gov. Multiple alignments for homology search performed using the Clustral W algorithm software and the phylogenetic tree was constructed (Hari prasad, 2016).

RESULTS AND DISCUSSION

Isolation, purification and identification of pathogen

Mixed infection of *Alternaria helianthi* and *A. alternata* was observed in all the three seasons k*harif, rabi* and summer . similar findings were reported by Prathuangwong *et al*. (1991).

The colonies of *A. helianthi* were identified morphologically by irregular colonies on potato dextrose agar (PDA), the colonies were initially hyaline which later turned grey colour in the centre. The centre of the colonies were picked up and purified on PDA by single spore isolation. The conidia of *A. helianthi* were born solitary, cylindrical to long ellipsoidal, straight or curved slightly, pale yellow to pale brown 2-10 septa (average of 5 septa), usually with transverse or occasionally with longitudinal septa and rounded at both the ends and measured 124.10 to 140.86 ìm (Fig.1a,b).

The colonies of *A.alternata* were identified morphologically by round regular colonies and were

dark brown in colour on PDA. The centre of the colonies were picked up and purified on PDA by single spore isolation. The conidia of *A. Alternate* were typically muriform, dark brown, thick walled and borne in long chains with 3 to 6 transverse septa and 0 to 3 longitudinal septa and measured 46.36 ìm (Fig. 2a,b). Morphological characters observed are in accordance with the findings made by Amaresh(1997) and Ahila Devi *et al*. (2016).

Pathogenicity

The culture of *A. Helianthi* was inoculated to 30 days old sunflower plants (KBSH 44) by spraying the conidial suspension (10^6 conidia /ml). The plants showed typical symptoms after eight days of inoculation as small scattered brown spots on the leaf surface. Later the spots increased in size, covering larger area with dark brown margin and

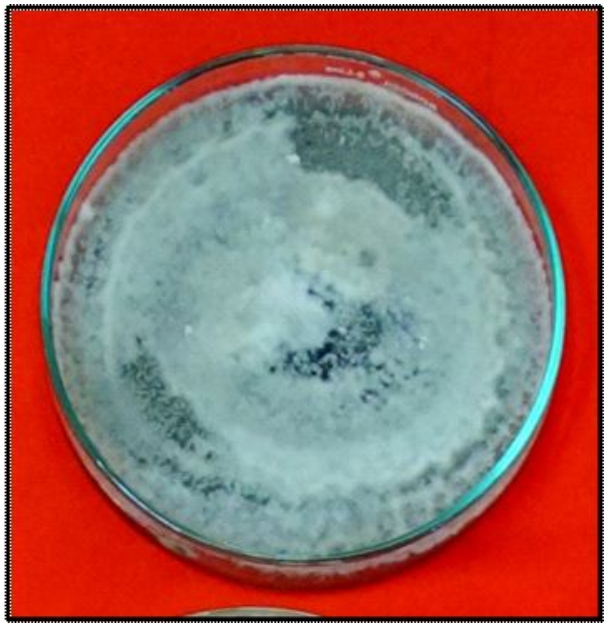


Fig. 1a: Colony of *A.helianthi*

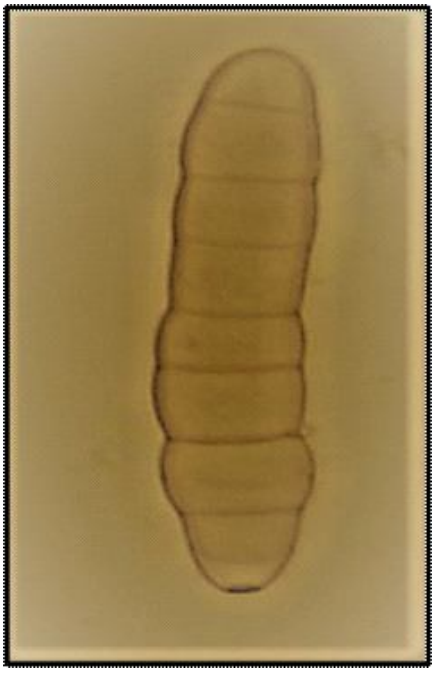


Fig. 1b: Conidium of *A. helianthi* (40x)

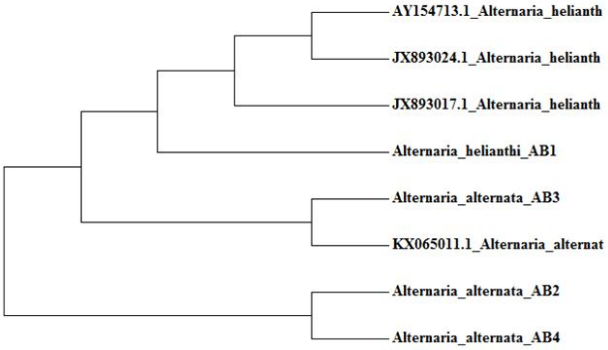
Fig. 2a: Colony of *A. alternata*

Fig. 2b: Conidium of *A. Alternate* (40x**)**

yellow halo with indistinct zonations (Fig.1a,b).

The culture of *A.alternata* was inoculated to 30 days old sunflower plants (KBSH 44) by spraying the conidial suspension (10^6 conidia/ml). The plants

1 SUB2900726 Alternaria_helianthi_AB1 MF563494
2 SUB2900741 Alternaria_alternata_AB2 MF563528
3 SUB2900747 Alternaria_alternata_AB3 MF563552
4 SUB2900750 Alternaria_alternata_AB4 MF563553



Phylogenetic tree analysis

showed typical symptoms 10 days after inoculation as small scattered brown spots on the leaf surface with dark brown margins (Fig.2a,b).

Molecular characterization
Isolation of genomic DNA by CTAB method

Genomic DNA of the fungus was isolated by CTAB method. The DNA obtained was observed by electrophoresis in 1.2 per cent Agarose gel. The DNA

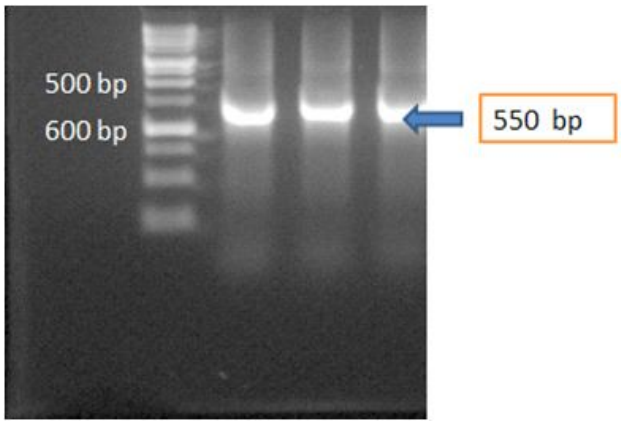


Fig. 3: ITS rDNA region amplification of *A. helianthi* and *A. alternata*

obtained was about 7000 bp approximate concentration of 91 çg/µg (Fig.3).

Amplification of ITS1 and ITS4 region

The full length ITS rDNA region was amplified with ITS2 primers for each isolate of *A. helianthi and A. alternata*. DNA amplicon was observed at region 550 bp with a concentration of around 150 ng/µg. The amplified products were checked by electrophoresis in 1.4 per cent Agarose gel (Fig. 3).

ITS rDNA sequence

The DNA sequences were obtained for ITS rDNA and were compared using bioinformatics tools like NCBI (National Centre for Bioinformatics) BLAST programme. Based on rDNA sequence comparisons, the identification of the isolates were confirmed as *A. helianthi* and *A. Alternate* .Similarly Kadam *et al*. (2009) clustered eight species of *Alternaria* into two major group's as *A. macrospora* and *A. helianthi* based on internal transcribed spacer (ITS) sequence ribosomal DNA (rDNA) and Phylogenetic relationship.

ITS rDNA sequence of *Alternaria helianthi.*and *A. alternate*were deposited in NCBI with GenBank accession numbers.

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