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MORPHOLOGICAL AND MOLECULAR VARIABILITY IN ISOLATES OF *Alternaria* Spp. CAUSING LEAF BLIGHT OF COTTON

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ABSTRACT
Leaf blight of cotton caused by *Alternaria* spp is an important disease and the samples were collected during the survey for the study of morphological and molecular variability. The study of variability with respect to morphological characters revealed that out of 25 isolates of *Alternaria* spp, 18 isolates showed complete resemblance with *Alternaria macrospora* and remaining 7 isolates resembles to *Alternaria alternata*. The conidial size varied from 42.43 x17.69 ìm in Garag and 76.20x13.20 ìm Tadakod isolates. The molecular variability study revealed that the DNA sequences of twenty five isolates were compared using the bioinformatics tool of the National Centre for Bioinformatics (NCBI) blast programme. Based on sequence comparison, the identification of *Alternaria* spp. isolates was confirmed and all the sequences of isolates were confirmed as eighteen isolates *Alternaria macrospora,* and seven as *Alternaria alternata* and accordingly phylogenetic trees were constructed. The present investigation revealed that there is greater morphological and molecular variability among the different isolates of *Alternaria* sp. It also shows that the genetic variation among the pathogen varies from location to location.

INTRODUCTION

Cotton is one of the most ancient and important fibre crops next only to food grains and is the principal raw material for a flourishing textile industry. Cotton known as the 'King of fibre' and called as 'White Gold' and is the most vital crop of commerce to many countries including India having largest cotton area of 10.5 million hectares with a production of 33.4 million bales and productivity of 568 kg/ha568. Karnataka state has an area of 4.64 lakh hectares and a production of 19.9 lakh bales bales with productivity of 769 kg/ha (Anon., 2017). The low productivity of cotton in Karnataka is attributed to many factors, one of which is the losses due to diseases. Among all the foliar diseases, Alternaria leaf spot disease has the prime importance. Conidiophores of *Alternaria macrospora* Zimm. arise singly or in groups. They are erect, simple straight or flexuous, cylindrical or tapering towards the apex and septate. They are pale brown in colour, 4-9 ìm thick and up to 180 ìm in length. Conidia are solitary or in chains of two, straight or curved, obclavate or with the body of the conidium ellipsoidal, tapering to a narrow beak and equal in length or up to twice as long as body. They are reddish brown in colour with four to nine transverse septa and several longitudinal septa (Ellis, 1971). Several attempts are made to classify *Alternaria* genera, several re-descriptions and revised criteria of these genera resulted in a growing number of new species. Results of a lifetime study on *Alternaria* taxonomy based upon morphological characteristics were summarised by Simmons (2007), in which 275 *Alternaria* species were recognised. Though, several cotton varieties and hybrids are being released from time to time, none of them has shown absolute resistance to this disease. This indicates the existence of variability among the isolates of this pathogen. Therefore, the present study was undertaken to understand the different aspects of *Alternaria* spp. with respect to its morphological and molecular variability. But not much work has been done on these aspects in the past. In addition, it helps in comprehensive understanding of the causal organism.

MATERIALS AND METHODS

During the survey,the cotton leaf sample infected by *Alternaria* spp. with typical dark brown, circular to irregular spots were collected from different cotton growing areas and were isolated from these infected leaves by standard tissue isolation technique in the laboratory. The single spore isolation of *Alternaria* spp was carried out by following the standard procedure.

Ten milli litre of clear, filtered two per cent water agar was poured into sterile petriplates and allowed to solidify. The dilute spore suspension was prepared in sterilized distilled water from 15 days old culture. One milli litre of such suspension was spread uniformly on agar plate. These plates were incubated at 27 ± 1ºC for 12 hrs. Then such plates were examined under microscope to locate single isolated and germinated conidium and marked with ink on the surface of the plates. The growing hyphal tip portion was transferred to PDA slants with the help of cork borer under aseptic conditions and incubated at 27 ± 1ºC. These culture tubes were used

for further studies. The pure culture of the fungus was obtained after ten to fifteen days of inoculation which showed whitish growth at initial stage turning later to ash grey colour. Such pure culture obtained was again sub cultured in Potato Dextrose Agar (PDA) slants and kept in the refrigerator at 5°C for further studies.

Proving the pathogenicity

Cotton seeds of hybrid Bunny Bt, were surface sterilized with 0.1 per cent Sodium hypochlorite and sown in earthen pots containing sterilized soil. They were allowed to grow for a month. Prior to inoculation, the plants were exposed to 95 per cent humidity for 24 hours. Thereafter, they were inoculated with spore suspension of (5.4×10^6 spores/ ml) the fungus, by using atomizer. After inoculation, the plants were exposed in the same conditions for 24 hours. Suitable control plants were maintained by spraying of sterile distilled water. After fifteen days of inoculation, the leaves exhibited initial symptoms of infection. Small, dull to dark brown, circular or irregularly shaped spots varying in diameter from 0.5 to 10 mm were observed. They developed concentric rings and presented a target board appearance which is better defined on the upper surface. The spots coalesced and occupied larger area of the leaf. The isolates were re-isolated and the morphological characters of the re isolated organisms were compared with the original culture. Hence, the causal agents of the disease were confirmed as different *Alternaria*.

The fungus was sub cultured on Potato Dextrose Agar (PDA) slants and allowed to grow at 27 ± 1°C for 15 days. These slants were then preserved in the refrigerator at 5°C and sub-cultured once in two months. This pure culture was used for further studies.

Morphological characters such as length and width of conidia, number of horizontal and vertical septa and beak length were measured under 40x using DIC (Differential Image Contrast) microscope and the pathogen was cultured on Potato Dextrose Agar. All the above mentioned measurements were compared.

Molecular variability among the different isolates

The mycelium collected from the cultures of *Alternaria* spp. after 5 days of incubation was used for DNA isolation. Total genomic DNA from fungal isolates were extracted by following Hi media kit protocol.

Internal Transcribed Spacer (ITS) was used to detect the variation among the ten isolates of *Alternaria* spp. collected from different districts of northern Karnataka. ITS1 and ITS4 Universal, *Alternaria macrospora* and *Alternaria alternata* specific primers obtained from Operon technologies, M/s Bangalore Genie, were used to determine molecular variability between the isolates. Polymerase Chain Reaction (PCR) amplification was done using Universal fungus ITS and

Primers

Organism	Primer code	Sequence	Size of amplified product
Universal fungus ITS	ITS1	5' TCC GTA GGT GAA CCT GCG G 3'	560 bp
	ITS4	5' TCC TCC GCT TAT TGA TAT GC 3'	
Alternaria macrospora	AmF	5′ CGGTACTACTGTCATCTTCG 3'	442bp
	AmR	5′ CTTACGGTACCTGAGTTGAC 3'	
Alternaria alternata	Aa F2	5' TGCAATCAGCGTCAGTAACAAAT 3'	380 bp
	Aa F3	5' ATGGATGCTAGACCTTTGCTGAT 3'	

Reaction mixture

Reagents	Volume/tube (ml)	
Template DNA	2	
Taq assay buffer (10x) (with $MgCl_2$)		
dNTP mix	1	1
Forward Primer- F	1	1
Reverse Primer- R		
Taq Polymerase		
Sterile distilled water	12.7	12.7
Total	20	

Alternaria specific primers.

ITS-1-5' TCC GTA GGT GAA CCT GCG G 3'

ITS-4 -5' TCC TCC GCT TAT TGA TAT GC 3' , Size of amplified product = 560 bp

Later, amplified PCR product was sent for sequencing to Chromous Biotech Pvt limited Bengaluru. PCR amplification of rDNA sequences for *Alternaria* spp. was conducted in ml reaction volumes using following primers and the reaction mixture mentioned below.

The PCR protocol was standardized to amplify rDNA sequences from a strain each of *Alternaria* spp. infecting cultivated species of Bt cotton. Negative controls were used to test for false priming and amplification. A 10 ml PCR amplification product for each of the *Alternaria* species was visualized on a 1 per cent agarose gel and viewed under UV light following staining with ethidium bromide.

The PCR protocol was standardized to amplify rDNA sequences from a strain each of *Alternaria* spp. infecting

PCR programme for amplification of 16S rDNA

Step	Universal fungus ITS		*Alternaria macrospora*		*Alternaria alternata*	
	Temp (°C)	Duration (min)	Temp (°C)	Duration (min)	Temp (°C)	Duration (min)
Initial denaturation	94	4	94	5	94	2
Denaturation	94	1	94	1	94	30 sec
Annealing	55	1	55	3	55	30 sec
Extension	72	1	72	2	72	30 sec
Final extension	72	20	72	10	72	5
Hold	4	30	4	30	4	30
Number of cycles:						
Denaturation	35		30		35	
Annealing						
Extension						

cultivated species of Bt cotton. Negative controls were used to test for false priming and amplification. A 10 ml PCR amplification product for each of the *Alternaria* species was visualized on a 1 per cent agarose gel and viewed under UV light following staining with ethidium bromide.

RESULTS AND DISCUSSION

The morphological study of causal organism revealed that, the conidia of different isolates were septated by 1-3 vertical and 1-5 horizontal septa. Isolate A_5, A_8, A_{14}, A_{15}, A_{16} showed maximum horizontal septa and isolates A_3 and A_{16} showed maximum vertical septa. A_7 showed maximum size of the conidia and least shown by A_4 and highest beak length by isolate A_8 and least length by $A_{20.}$ Table 1 showed that, conidia of different isolates were septated by 1-3 vertical and 1-5 horizontal septa. The isolates, A_5, A_8 , A_{14} , A_{15} and A_{16} showed maximum horizontal septa of 5 whereas, minimum horizontal septa (1) was observed in the isolates, A_{16} and A_{17}. The isolates, A_3 showed maximum of 3 vertical septa and isolates, A_1, A_2 , A_4, A_5, A_7, A_8, A_{10}, A_{17}, A_{19} and A_{20} showed minimum of 1vertical septa. The isolates, A_7, A_{11} and A_8 showed maximum size of 76.20x13.20 ìm, 74.36x17.40 ìm and 73.89x 15.69ìm, respectively. The least size of the conidia (42.43 x17.69 ìm) was observed in isolate, A_4. By comparing with *A. macrospora* as per structural figure described by Ellis (1971) revealed that out of 25 isolates, 18 isolates showed complete resemblance with *Alternaria macrospora* and remaining 7 isolates resembles to *Alternaria alternata* morphologically which is in accordance with the findings of Anil (2013) who reported that out of their 12 isolates, only four showed complete resemblance with *A. macrospora*, morphologically. Similarly Jadhav et al. (2011) and Ramegowda (2007) observed diversity in cultural and morphological characteristics such as growth rate, type of growth, colony colour and sporulation among different isolates of *Alternaria* spp., in cotton.

The PCR protocol was standardized to amplify rDNA sequences from a strain each of *Alternaria* spp. infecting cultivated species of Bt cotton. Negative controls were used to test for false priming and amplification. A 10 ml PCR amplification product for each of the *Alternaria* species was visualized on a 1 per cent agarose gel and viewed under UV light following staining with ethidium bromide.

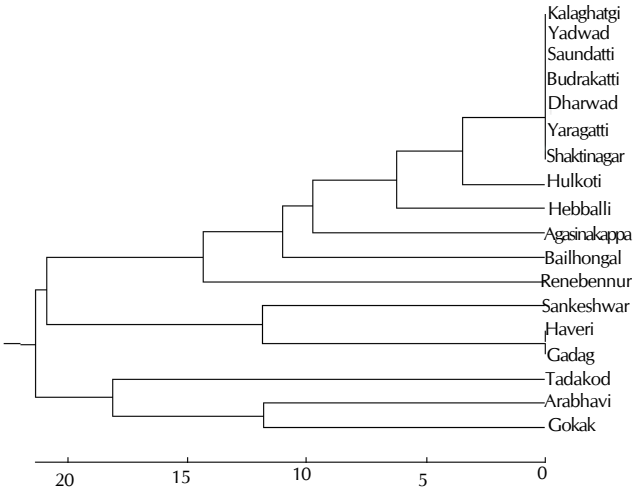
The DNA sequences of twenty five isolates were compared using the bioinformatics tool of the National Centre for Bioinformatics (NCBI) blast programme. Based on sequence comparison, the identification of *Alternaria* spp. isolates was confirmed and all the sequences of isolates were confirmed as eighteen isolates *Alternaria macrospora,* and seven as *Alternaria alternata* and accordingly phylogenetic trees were constructed (Fig. 1 & 2).

Polymerase chain reaction (PCR) based molecular markers are useful tools for detecting genetic variation within populations of phytopathogens. PCR amplification of *Alternaria* spp. with conserved primers ITS1 and ITS4 yielded an approx. 560 bp rDNA amplicon product. Based on sequence comparison, the identification of *Alternaria* spp. isolates were confirmed and all the sequences of isolates were confirmed as eighteen *Alternaria macrospora* and seven *Alternaria*

Table 1: Morphological variability of different isolates of *Alternaria* spp

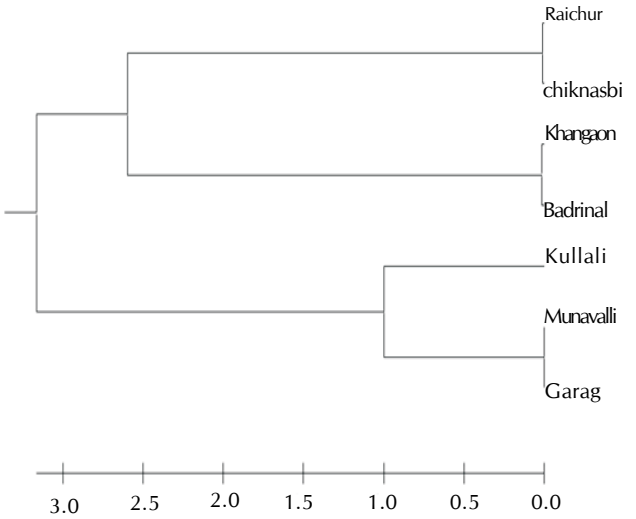
Isolates	Size of conidia (μm)(Length X Breadth	Septation		Beak length(μm)
		Vertical	Hrizontal	
A_1	46.85 x16.70	1	3	14.88
A_2	50.83 x28.07	1	4	21.81
A_3	61.98 x30.08	3	4	17.40
A_4	42.43 x17.69	1	3	23.45
A_5	57.71 x 14.67	1	5	22.45
A_6	56.52x 12.33	2	4	21.46
A_7	76.20x13.20	1	4	23.28
A_8	73.89x 15.69	1	5	28.64
A_9	44.75x11.44	2	4	19.84
A_{10}	48.36x11.96	1	4	16.41
A_{11}	74.36x17.40	2	3	21.39
A_{12}	69.25x16.32	2	4	23.30
A_{13}	72.73x12.05	2	4	26.03
A_{14}	44.73x17.39	2	5	21.35
A_{15}	57.35x13.78	2	5	21.34
A_{16}	65.56x 16.34	3	5	24.35
A_{17}	62.36x18.61	1	3	23.25
A_{18}	63.58x19.31	2	2	24.01
A_{19}	48.07x14.91	1	1	20.90
A_{20}	59.05x20.52	1	1	11.79

A-1 = Khangaon	A-2 = Badrinal	A-3 = Dharwad	A-4 = Garag
A-5 = Budrakati	A-6 = Tadkoad	A-7 = Bailhongal	A-8 = Yargatti
A-9 = Saundatti	A-10 = Arabhavi	A-11 = Gokak	A-12 = Sankeshwar
A-13 = Khalghatgi	A-14 = Munnavali	A-15 = Kuloli	A-16 = Shaktinagar
A-17 = Mantralaya	A-18 = Agasinkoppa	A-19 = Yadwad	A-20 = Chiknasbi



(There was 3 major clusters among 18 different isolates of *A macrospora* indicated that there may be existence of variability in *A macrospora* with respect to races or species level)

Figure1: Phylogenetic tree for *Alternaria macrospora* of different isolates



(There was 3 major clusters among 7 different isolates of *A alternata* indicated that there may be existence of variability in *A alternata* with respect to races or species level)

Figure 2: Phylogenetic tree for *Alternaria alternata* of different isolates

alternata.

The present investigation revealed that there is greater molecular variability among the different isolates of *Alternaria sp*. It also shows that the genetic variation among the pathogen varies from location to location. Findings of this experiment are in agreement with the results of Jadhav *et al*. (2011) who conducted molecular variability test among different isolates of *Alternaria macrospora* and found that all the primers showed the polymorphism in the range of 64-92 per cent; however maximum polymorphism was observed by OPB-9 primer.

Finally, involvement of *Alternaria* spp in causation of leaf blight disease was confirmed through pathogenicity test. The phylogenetic tree representation showed that, among 18 isolates of *A.macrospora*, there is variation in different cluster of isolates indicted that there may be variability with respect to races or species level. There was 3 major clusters among 18 different isolates of *A macrospora* indicated that there may be existence of variability in *A macrospora* with respect to races or species level (Fig.1). Similar variability was observed in 7 isolates of *A. alternata*. There was 3 major clusters among 7 different isolates of *A alternata* indicated that there may be existence of variability in *A alternata* with respect to races or species level (Fig. 2).

Anyhow, finally observed that *A.macrospora* is the major species of *Alternaria* causing leaf blight in cotton and in some cases there may be involvement of *A. alternata* causing leaf blight. This is in agrrement with findings of Sangeeta et al (2016) who conducted molecular variability test among different isolates of *Alternaria* spp causing leaf blight in cotton. The primer pAmac however, was not specific to *A. macrospora* of cotton but supported amplification of the rDNA fragment from several species of *Alternaria* (Kadam, 2005).

Molecular techniques, if not alone, can be used in conjunction with classical methods where the latter approaches can at least narrow pathogen diagnosis to genus level. Once genus is narrowed by morphology, symptomatology, host-specificity, *etc.*, then PCR can be used to differentiate species. The outcome of the result finds the way for the exact cause or species involved and also the existence of pathogenic variability among isolates of different locations. So that effective mode of management of leaf blight disease strategy can be developed in future.

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