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Assessment of incidence and characterization of pathogen associated with *Alternaria* leaf blight of cotton in Tamil Nadu

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Abstract
Cotton is a natural fibre and a key cash crop farmed in over 50 nations worldwide. *Alternaria* Leaf Blight (ALB) incited by the *Alternaria* spp is the most serious disease which inflicts yield loss up to 37 percent. Roving survey was done to evaluate the status of ALB in nine different cotton growing areas in Tamil Nadu between2020 to 2022. The mean district incidence of ALB incidence in surveyed districts ranged from 6.60 to 12.71 PDI (Percent Disease Index). Dindigul district had the highest average district incidence (12.71 PDI), while Trichy district had the lowest average incidence (6.60 PDI). A total of 10 isolates were isolated from the ALB infected cotton field and morphological characterization was done. The colonies of these isolates showed light to dark brown colour with concentric zone. The conidia colour varied from pale to dark brown colour and the size ranged from 26.33-38.19 µm to 8.12-14.12 µm with transverse (1-6) and longitudinal septa (1-3). The isolate CA3 was proved to be the most virulent as it was found to produce the highest lesion of 3.7 cm (size) within 6 days in detached leaf assay. The molecular characterization of isolates were done by PCR amplification of ITS region by using universal primers and all the isolates were amplified at 560 bp. Morphological and molecular characterization confirmed all the isolate as *Alternaria* spp . Sequencing and blast analysis confirmed the isolate CA3 as *A. alternata.*

Keywords: *Alternaria* leaf blight, cotton, leaf detached assay, molecular characterization, survey

Introduction
Cotton is the most essential fibre crop in the world comprising fifty percentage of the industry's total use of fibres. Cotton is the undisputed king of fibres. Cotton is one of the agricultural products that provide both natural fibre and edible oil. It is an agro industrial crop grown in both developing and industrialised nations (Bedane and Arkebe, 2019) [22]. Globally cotton was cultivated in an area of 31.66 million hectares with production and productivity of 113.11 million bales and 778 kg/ha respectively. The world's largest cotton grower is India. India produced 371.00 lakh bales of cotton from 12.97 million hectares, with productivity of 487 kg/ha (Cotton Outlook Report- January to May 2021, Cotton Corporation of India). It is an important crop for the Indian economy and subsistence of cotton farmers. Over 60 million people are employed by the cotton industry in India. Numerous biotic and abiotic stressors have an effect on cotton crop yield. The most significant and recurring disease is the *Alternaria* Leaf Blight (ALB), caused by *Alternaria* spp, which drastically reduced the cotton production (Cui *et al.*, 2000) [24}. Initially reported in the United States in 1918, the disease has now disseminated to all cotton-growing regions worldwide (Faulwetter, 1918) [23]. It is characterized by small, brown, circular lesions on the leaves that are surrounded by distinct purple margins. Similar symptoms can be observed on buds, flower and bolls in mature plants. As the disease progresses, the lesions expand and become dry and grey in the centre, exhibiting shot-hole symptoms on leaves. These spots coalesced and occupy large leaf areas resulting in severe defoliation, affecting cotton yield and quality (Watkins 1981) [25]. In cotton, up to 26 percentage of yield reduction due to *Alternaria* leaf blight was recorded by Chattannavar *et al.*, (2006) [2]. Yield loss up to 37 percent was observed by Padaganur *et al.*, (1989) [28] in India. In light of this, the current investigation was carried out to identify the endemic or hot spot area and assess the severity of ALB of cotton in Tamil Nadu. Morphological and molecular characterization of pathogen associated with the disease was studied.

This will aid researchers and extension workers in directing their efforts towards regions with the highest incidence of the disease. By identifying these hot spots, resources can be allocated strategically for the sustainable management of the disease, optimizing the effectiveness of control measures and ensuring better outcomes for cotton cultivation.

Materials and Methods

Assessment of *Alternaria* leaf blight incidence in major cotton growing regions of Tamil Nadu

A roving survey was conducted in nine cotton-growing districts of Tamil Nadu, namely Coimbatore, Dindugal, Virudhunagar, Thoothukudi, Tirunelveli, Trichy, Salem, and Perambalur, to assess the prevalence of *Alternaria* leaf blight (ALB) in the region. In each district, a minimum of three villages were selected, and from each village, five fields were chosen for the study. A total of 50 plants were randomly selected from each field, and the severity of the disease was evaluated on 20 leaves per plant using the disease score chart (0-4 grade) described by Sheo Raj (Raj, 1988) [18]. The characteristic symptoms of ALB observed were also recorded during the survey. The following formula was used to calculate percent disease index.

$$\text{Percent disease index (PDI)} = \frac{\text{Sum of individual ratings}}{\text{Total number of leaves observed}} \text{ X } \frac{100}{\text{Maximum grade}}$$

Isolation of *Alternaria* leaf blight pathogen

Cotton plants showing characteristic symptoms of ALB were collected from various locations of Tamil Nadu and brought to the laboratory. The samples were subjected to a thorough washing using sterile distilled water. Along with some healthy tissue, the diseased parts were sliced into 2-3 mm pieces and surface sterilised with 0.1 percent sodium hypochlorite (NaOCl) for 30s before being rinsed three times in a series of sterile distilled water. The leaf pieces were wiped dry using a paper towel before plating on Potato Dextrose Agar medium (PDA) on Petri plates with a pinch of Streptomycin Sulphate. Plates were incubated at 25 °C for 8-10 days. Emerging mycelial colonies were purified by hyphal tip method (Dhingra and Sinclair, 1985) [4] and the pure cultures were maintained on PDA slants for further studies.

Identification of the *Alternaria* leaf blight pathogen (*Alternaria* spp)

Pure culture maintained on PDA medium was used for studying morphological characters *viz* colour, mycelial character, shape and size of conidia. The morphological characters of conidia were studied by collecting spores from pure culture of the fungal isolates.

Pathogenicity assay

Inoculum preparation

The S medium composed of 20 g sucrose, 30 g $CaCo_3$ and 20 g of agar per litre of water (pH 7.4) was used to induce sporulation. The isolates were grown on S medium and incubated at room temperature (28±2 °C) for 4-5 days. Each plate was added with ten millilitres of sterile distilled water. Mycelium along with spores were scraped off from the surface of the medium using sterile spatula and transferred to 50 ml falcon tubes with sterile distilled water. It was filtered through muslin cloth and the spores were collected. A haemocytometer was used to adjust the spore concentration to $5x10^5$ spores per ml after the spores were suspended in sterile distilled water.

Glass house experiment

Earthen pots of 30 cm diameter were filled with sterilized potting mixture and placed in glass house. The potting mixture consists of red soil + sand + FYM (2:1:1) was sterilized in an autoclave at 121 °C at 15 psi for 2 hrs for two consecutive days. Seeds of cotton variety CO17 were sown in the pot @ 2 / pot. The spore suspension containing $5x10^5$ spores/ml was inoculated on 30 days old plants. Before inoculation, healable wounds were created by using carborundum dust. The inoculated plants were frequently inspected for symptom expression. On symptom development, reisolation of pathogen was done and its identity was confirmed.

Detached leaf assay under *in vitro* condition

The second or third oldest leaves of the cotton variety CO 17 were plucked from one month old seedling and cleaned with distilled water. The leaves were placed in a Petri plate containing moist absorbent cotton. A sterile needle was used to make a pin prick in the leaves, and the leaves were then infected with two to three drops of conidial ($5x10^5$ spores/ml) suspension. Sterile distilled water without conidial suspension served as control. The plates were incubated at room temperature (28±2 °C) for seven days and the diameter of the lesion was measured.

Molecular characterization DNA

PCR amplification of the Internal Transcribed Spacer (ITS) region of the rDNA

CTAB method was used to extract the DNA, after the pathogenic isolates had been cultured in potato dextrose broth for 15 days. Amplification of ITS regions in isolates was carried out by using a universal primer ITS1 and ITS4. PCR was performed with a reaction volume of 10 µl and the reaction cycle consisted of 60 seconds at 94 °C for denaturation, 45 seconds at 53 °C for annealing, and 90 sec at 72 °C for extension (Mohammadi & Bahramikia, 2019) [10].

ITS1 (5'-TCC GTA GGT GAA CCT GCG G-3')

ITS4 (5'- TCC TCC GCT TAT TGA TAT GC-3')

Agarose gel electrophoresis

Based on the procedure outlined by Sambrook *et al*., (1989) [15], agarose gel electrophoresis was carried out to assess the DNA's purity and to separate the products of the polymerase chain reaction. The gel was photographed and analysed using an ultraviolet transilluminator. The PCR products sizes were evaluated by comparing them to a standard 1kb ladder (Bangalore Genei Pvt. Ltd., Bangalore, India).

Results and Discussions

Incidence of *Alternaria* leaf blight of cotton in Tamil Nadu (2020-22)

Roving survey was conducted in nine cotton growing districts

viz., Coimbatore, Dindigul, Virudhunagar, Salem, Trichy, Tiruppur, Perambalur, Thirunelveli and Thoothukudi in Tamil Nadu during 2020-2022 to assess the incidence of ALB. A total of 47 villages were surveyed, among the villages, the maximum incidence of 17.3 PDI was recorded in Vellamaruthupatti village in Dindigul district followed by Guvayanayakkanpatti village (17.0 PDI) of at the same district. The lowest PDI of was observed in Keeranatham village (5.0) of Coimbatore district. The typical symptoms *viz* small, irregular or circular, pale to brown spot encircled by concentric rings was observed on the infected plants. Later the leaves turn brittle and drop off. On the bracts and bolls dark brown spot with concentric rings were observed. Similar symptoms were reported by Jadhav *et al.*, (2011) [7] and Chohan. S *et al*., (2020) [3]. The mean district incidence of ALB during 2020-2022 was ranged from 6.60 to 12.71 PDI. The highest district average incidence of 12.71 PDI was found in Dindigul and the lowest PDI of 6.60 was recorded in Trichy district. This was in agreement with the findings of Vairavan *et al*., (2021) [19] who recorded *Alternaria* leaf blight incidence of 19.8 PDI at Arasappapillaipatti village of Dindigul district. In Tamil Nadu, the prevalence of leaf blight disease increased to 54.62 PDI during 2013-2014, resulted in 32 per cent yield loss (Saravanan *et al*., 2015) [27]. *Alternaria* leaf blight incidence of 13.5 per cent was recorded in Nandod taluka of Narmada district, Gujarat (Prashant *et al*., 2017) [26]. Chattannavar *et al*., (2010) [2] observed ALB incidence of 19.00 per cent in the Dharwad district of Karnataka.

Phenotypic characterization of the pathogen

Ten pathogenic isolates were collected from infected samples of different cotton growing districts in Tamil Nadu. Morphological characterization was done for all the 10 isolates. The colony colour of these isolates was observed to be dark to light brown with raised or flat texture with concentric zone. The margin of the colonies was smooth or irregular. These findings were in consistent with Barnett and Hunter's early descriptions of the genus *Alternari*a infecting cotton in 1972. Sampathkumar and Raghavendra, (2023) [16] reported that colony characters of *Alternaria* exhibited variations in colour, ranging from grey to ashy grey, whitish grey, and blackish grey. The margins of the colonies were observed to be smooth or irregular, while the colony texture varied from velvety to rough, with mycelial growth appearing flat or raised. The *Alternaria* spp infecting cotton showed pale to dark brown colonies with regular or irregular growth pattern. The mycelium was septate with muriform shape of conidia having many septations (Rajesha *et al*., 2020) [14]. The margin colour of *Alternaria* spp varied from light grey or light brown and whitish pink, regular to irregular margins with flat to raised texture (Anil *et al*., 2017) [1]. The colony colour of the *Alternaria* isolates obtained from the cabbage were brown, light grey or olivaceous grey (Ogada *et al*., 2021) [11].

The phenotypic characters of conidia *viz* length, width, shape and colour of the conidia were observed for all the ten isolates. The colour of the conidia varied from pale brown to dark brown. The length and width of the conidia ranged from 26.33-38.19µm and 8.12-14.12µm respectively with 1- 4 transverse and 1- 3 vertical septum. The lengthiest conidia were observed in the isolate CA 3 (38.19µm) and the shortest (26.33µm) was observed in CA 4. This was in agreement with the findings of Ellis (1971) [5]. Sangeetha *et al*., (2016) [17] who found the size of the *Alternaria* conidia ranged from 42.43-76.20 x 11.44-30.08 µm with 1-3 vertical, 1-5 horizontal septation. Jadhav *et al*., (2011) [7] observed that the size of conidia ranged from 20.81-56.23 x 9.2- 27.10 µm with 1 to 6 transverse and 0 to 4 longitudinal septa. The morphological variability of the *A.macrospora* was studied by Waghunde *et al*., in 2018 [20] found that the conidia size ranged from 42.46-70.11x 10.66-21.84 µm with the 0 - 3 longitudinal and transverse septa. Based on the mycelial and conidial characters, all the 10 isolates were identified as *Alternaria* spp.

Pathogenicity

The pathogenicity of the *Alternaria* isolates was proved on cotton cultivar CO 17 grown in glass house under pot culture. After the artificial inoculation, the symptoms development took 9-10 days for its expression. The symptoms produced were similar to the symptoms observed in field. The pathogen were re isolated and it identity was confirmed thus proving the Koch's postulates. Similarly, pathogenicity of ALB was demonstrated in numerous cases utilising the artificial inoculation method under glass house. Upland cotton Acala 1517-08 and Pima cotton DP 348 inoculated with *A. alternata* isolates, showed disease severity indexes ranging from 26.0 to 65.0 and 46.0 to 80 (Zhu *et al*., 2019) [21]. Olmez *et al.*, (2023) [12] found that symptom expression of ALB took 20 days after artificial inoculation with conidial suspension (10^6 /ml) and the symptoms observed were similar to the field symptoms. *Alternaria* isolates inoculated at 2 to 3 leaf stage expressed symptoms on cotton cultivar MCU5 after 8 days of inoculation (Rajesha *et al*., 2020) [14].

The virulence of the isolates was studied by using the detached leaf assay. The virulence of the isolates was determined by measuring the lesion size. The characteristic symptoms expressed after 6-7 days of inoculation. The lesion colour varies from brown to blackish brown and the diameter varied from 0.5 to 3.7cm. The highest lesion size of 3.7 cm (dia) was produced by the isolate CA 3 and it was selected as the most virulent isolate. The least lesion size of 0.5cm (diameter) was observed in the isolate CA 8. Rajesha *et al*., (2020) [14] observed brown lesion on the leaves inoculated with *Alternaria* spp which is in corroboration with our present findings.

Molecular characterization of *Alternaria* isolates

In terms of molecular ecology, ITS region of fungi's DNA has undergone significant sequencing; it has been proposed as the official fungal barcode sequence. PCR amplification was done with rDNA region of all the 10 isolates was using universal primers of ITS1 and ITS4. All the 10 isolates showed the expected specific amplicon size of 560bp which depicts molecular based confirmation of *Alternaria* spp. The amplicon of the most virulent isolate CA3 was sequenced and the nucleotide BLAST analysis was performed in the NCBI. The results revealed that the DNA sequence displayed 99 per cent homology with that of CA3 as *Alternaria alternata.* The sequence was submitted to NCBI (National Centre for Biotechnology Information) genebank, USA and the accession number was obtained (OR244396). The results were in line with those of Sampathkumar and Raghavendra (2023) [16], who observed that all of the *Alternaria* isolates produced 560 bp bands from their ITS primers.The genus-specific primers amplified multiple bands for all the isolates, while the *A. macrospora* specific primers (Am) and *A.*

alternata species-specific primers (Aa) consistently amplified at 442 bp and 320 bp, respectively, for all the isolates. Sequence alignments of the ITS (570 bp) and the TEF1 (470 bp) revealed a high level of genetic uniformity within the presumed *A. alternata* population (Le *et al*., 2019) [9].

Table 1: Disease score chart for *Alternaria* leaf blight in cotton

Disease grade	Descriptions
0	Immune, No infection, completely free from disease.
1	Highly resistant, Few <2 mm, scattered brown spots and 0.1 to 10% infected leaf area covered.
2	Moderately resistant, Spots bigger, 3 mm, not coalescing, brown and 11-20% infected leaf area.
3	Moderately susceptible, Spots 3-5 mm, irregular in shape-coalescing, 21-40% infected leaf area.
4	Highly susceptible, Spot coalescing to form bigger lesions irregular >40 infected leaf area.

Table 2: Incidence of *Alternaria* leaf blight in major cotton growing districts of Tamil Nadu

S.No	District and villages	GPS co-ordinates	Percent Disease Index*
Coimbatore			
1	Kallipalayam	11.1600ºN,76.941ºE	5.3
2	Kovilpalayam	11.9548ºN,77.507ºE	7.6
3	Vellamadai	11.9527ºN,76.5914ºE	8.3
4	Thottipalayam	11.0439ºN,76.5532ºE	7.6
5	Samainayakkanpalayam	11.1653ºN,76.9863ºE	9.5
6	Keeranatham	11.1142°N,76.9938°E	5.0
7	Chenthampalayam	11.8161ºN,77.5356ºE	5.3
8	Vadapudur	11.0619°N,76.9327°E	8.5
Dindigul			
1	Guvayanayakkanpatti	10.2144ºN,77.5032ºE	17.0
2	Kariyagoundanpatti	10.2639ºN,77.4830ºE	14.3
3	Boothipuram	10.1437ºN,77.5267ºE	12.0
4	Ambaligai	10.5475°N,77.7257°E	7.5
5	Kamatchipuram	10.1568°N,77.7271°E	12.3
6	Govindhapuram	10.3704°N,77.9746°E	11.0
7	S,Paraipatti	10.3060°N,77.8757°E	10.5
8	Malaiyandipuram	10.1812°N,77.4959ºE	12.5
9	Vellamarathupatti	10.2814ºN,78.0120ºE	17.3
Virudhunagar			
1	Subramaniapuram	9.1550ºN,78.5485ºE	7.5
2	Shanmugapuram	9.1547ºN,77.5226ºE	8.3
3	Ramalingapuram	9.2614ºN,78.4983ºE	7.8
4	Sholapuram	9.0521ºN,78.0231ºE	7.5
5	Kariyapatti	9.6744°N,78.1029°E	6.5
6	Subbaiyapuram	**9**.2430°N,77.9392°E	7.3
7	Saminathapuram	11.0922°N,77.4318°E	6.7
8	Vadakarai	9.6524°N,78.0695°E	7.8
Thoothukudi			
1	Villicheri	9.2331ºN,77.4865ºE	8.0
2	Nalattumuthoor	9.3921ºN,77.4920ºE	7.3
3	Nalatin pudur	9.0805ºN,77.4937ºE	6.1
Tirunelveli			
1	Sankarankovil	9.1865ºN,77.5834ºE	7.0
2	Rastha	8.4911ºN,77,3911ºE	10.5
3	Manoor	8.8550ºN,77.6522ºE	7.4
4	Perumalpatti	9.3263ºN,77.5785ºE	15.1
Trichy			
1	Muthuvathoor	10.9772ºN,78.9720ºE	6.5
2	Kallagudi	10.9916ºN,78.9424ºE	7.3
3	Varakkuppai	10.9982ºN,78.9365ºE	6.0
Tiruppur			
1	Vellaigoundan valasu	10.6223°N,77.5118°E	11.5
2	Avinashi	11.1730°N,77.2686°E	9.3
3	Samiyanpalayam	11.4577°N,77.7220°E	6.5
Salem			
1	Thamukkapalaym	11.3064ºN,78.4521ºE	12.7
2	Ramanathapuram	11.2930ºN,78.4443ºE	11.7
3	Vazhapadi	11.3940ºN,78.2240ºE	13.0
Perambalur			
1	Krishnapuram	11.3878ºN,78.7827ºE	11.3

2	Nergunam	11.4323ºN,78.8390ºE	9.7
3	Veppanthattai	11.2151ºN,78.4815ºE	10.4
4	Varagupadi	11.0918 ºN,78.5424ºE	12.3
5	Alathur	11.0747 ºN,78.5024ºE	8.7
6	Venbavoor	11.3765ºN,78.8285ºE	11.3
		SE(d)	0.903
		CD (P= 0.05)	1.797

*Mean of three replications

Table 3: Cultural characters of *Alternaria* isolates

Isolates	Colony characters			
	Colony colour	Mycelia texture	Zonations	Types of margin
CA 1	Brown	Flat	Concentric	Smooth
CA 2	Brown with brownish white in centre of colony	Raised	Concentric	Smooth
CA 3	Blackish brown	Flat	Concentric	Irregular
CA 4	Brown	Raised	Concentric	Irregular
CA 5	Brown	Raised	No zonation	Irregular
CA 6	Olive brown	Raised	Concentric	Smooth
CA 7	Brown	Flat	Concentric	Smooth
CA 8	Brown with brownish white in centre of colony	Flat	No zonation	Irregular
CA 9	Blackish brown	Raised	Concentric	Smooth
CA 10	Brown	Flat	Concentric	Smooth

Table 4: Conidial characters of *Alternaria* isolates

Isolates	Size		Number of septation		Colour
	Length (µm)	Width (µm)	Transverse	Longitudinal	
CA 1	30.98	13.33	3	2	Dark brown
CA 2	33.10	11.42	3	2	Light brown
CA 3	38.19	8.35	4	3	Dark brown
CA 4	26.33	8.12	3	2	Pale brown
CA 5	37.91	9.62	2	1	Dark brown
CA 6	28.37	14.12	4	1	Light brown
CA 7	27.51	8.46	3	2	Light brown
CA 8	32.64	9.73	3	3	Dark brown
CA 9	35.31	8.45	3	2	Light brown
CA 10	28.64	8.56	3	2	Light brown

Table 5: Assessment of virulence of the *Alternaria* isolates (Detached leaf bioassay)

Isolates	Lesion size (cm) *	Days taken to express the symptoms
CA 1	2.8^{b}	7
CA 2	1.7^{d}	7
CA 3	3.7^{a}	6
CA 4	0.9^{fg}	7
CA 5	1.6^{de}	8
CA 6	1.2^{ef}	6
CA 7	0.7^{g}	8
CA 8	0.5^{g}	7
CA 9	2.4^{bc}	9
CA 10	2.2^{c}	7

SE(d)=0.2097 C.D. = 0.597

*Mean of three replications. In a column, any two means having a common letter is not significantly different at the 5% level of DMRT

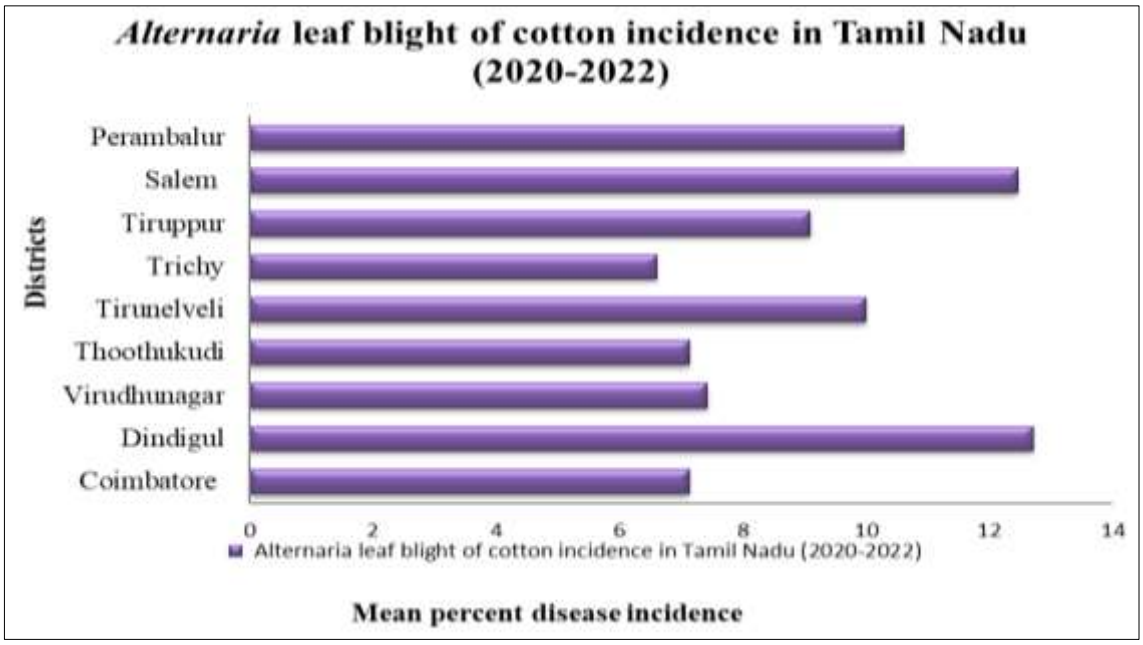

Fig 1: District wise incidence of *Alternaria* leaf blight of cotton in Tamil Nadu

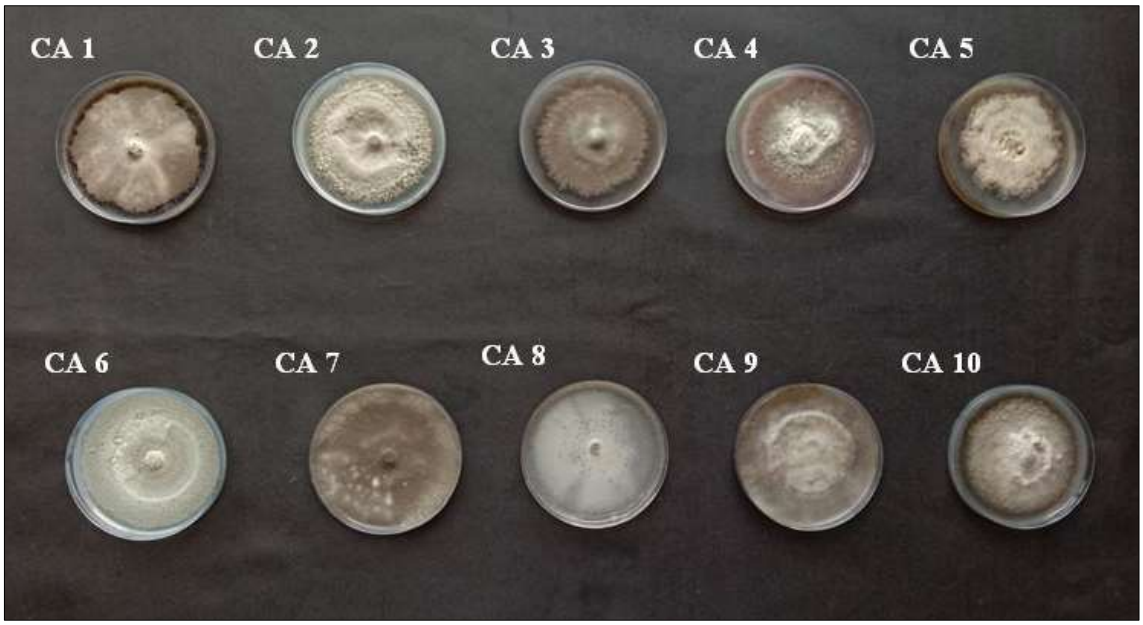

Fig 2: *Alternaria* isolates collected from cotton

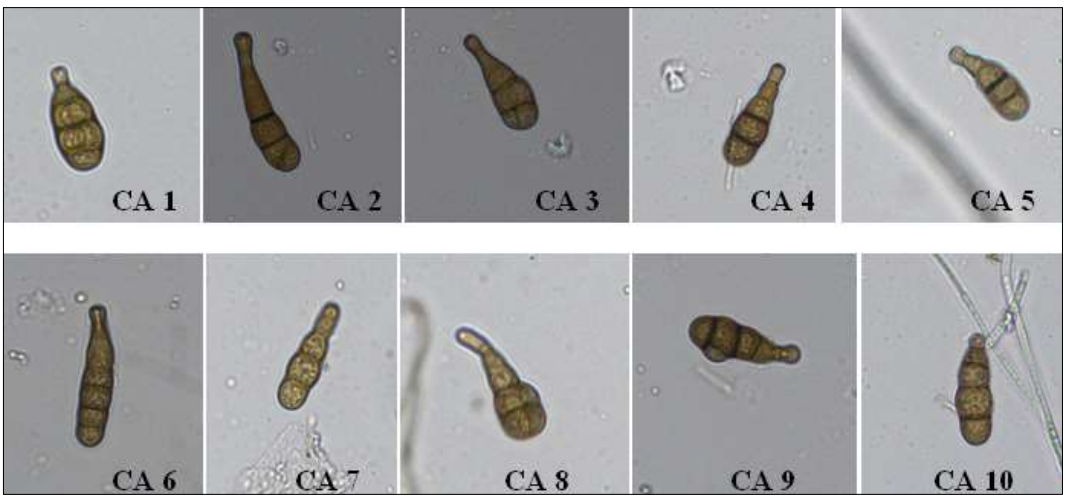

Fig 3: Conidia of *Alternaria* isolates collected from cotton

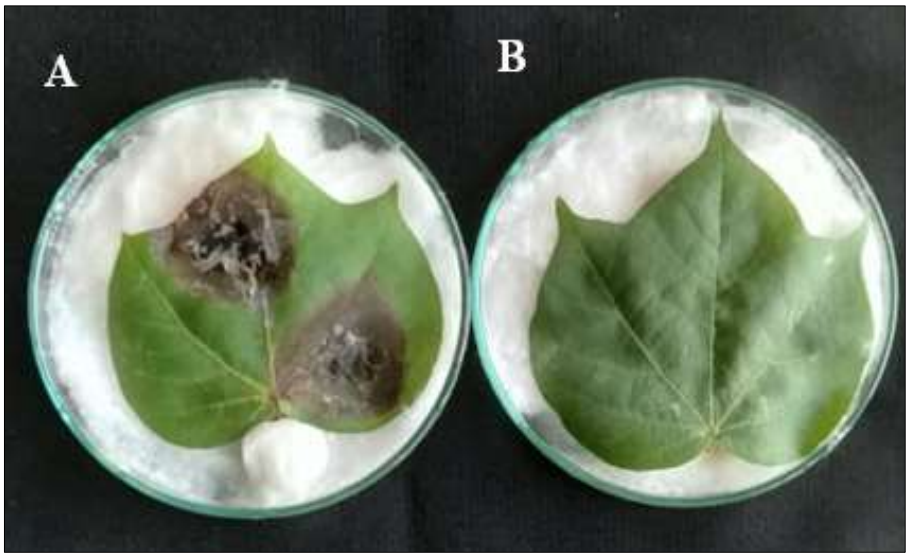

Fig 4: Leaf detached assay A. CA 3 B. Control

Conclusion

Alternaria leaf blight is one of the major foliar diseases in cotton. The continuous monitoring of cotton ALB is highly imperative to make timely decision for management of the disease to sustain the crop yield. In this work, we conducted a survey to determine the severity of ALB in important cotton growing areas in Tamil Nadu from 2020 to 2022. The data collected during this survey is useful to researchers and extension officials for identifying the hotspot area of ALB incidence. Globally diverse species of *Alternaria* found to be associated with ALB in cotton. In our study, we confirmed that the pathogen associated with the ALB in Tamil Nadu is *Alternaria alternata* through morphological and molecular characterization.

Future scope

Future research may concentrate on determining how *Alternaria* leaf blight prevalence and severity in Tamil Nadu are affected by shifting climatic trends. Effective disease management methods may be developed with the aid of an understanding of these dynamics. To detect *Alternaria* leaf blight and other cotton diseases, researchers may create more precise and speedy diagnostic methods. These developments could improve our knowledge of the disease's epidemiology.

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