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Morphological and Molecular Identification of Alternaria Alternata Causing Leaf and Fruit spot Symptoms on Sweet Orange from Tirupati District, Andhra Pradesh

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Abstract: Necrotic spots on leaves and fruits were observed on sweet orange trees during July and August of 2023 and 2024. Severely affected leaves and fruits exhibited premature drop. The pathogen was isolated from these spots, and its morphological characteristics were examined. The fungal colonies cultured on potato dextrose agar (PDA) displayed ashy black mycelium, with conidia arranged in chains featuring both transverse and longitudinal septa, leading to the identification of the pathogen as *Alternaria alternata*. Molecular analysis of the internal transcribed spacer (ITS) and translation elongation factor 1-alpha (TEF-1alpha) regions of the fungal isolate further confirmed its identity as *Alternaria alternata.* The pathogenicity of selected isolates was validated through the detached leaf assay technique. To the best of our knowledge, this represents the first documented case of *A. alternata* causing foliar and fruit spots, as well as blight, in the Tirupati district.

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1. Introduction

Citrus, belonging to the Rutaceae family, is a crop of considerable importance in horticulture on a global scale. It is cultivated not only in India but also in various tropical and subtropical regions around the world. India holds the position of the third-largest producer of citrus, with an annual output of 14.01 million metric tonnes (Gurjar *et al*., 2023). The appealing flavour and high vitamin content, including vitamins A, B, and C, contribute to the popularity of citrus among consumers. This fruit comprises several economically significant species and plays a crucial role in India's economy, ranking as the third

largest fruit industry in the country, following mango and banana. The most commercially cultivated citrus species in India include grapefruit, lemon, lime, sweet orange, and mandarin, with sweet orange being particularly noteworthy. Over the last four to five decades, Andhra Pradesh and Maharashtra have been the leading states for sweet orange cultivation in India. Following the division of the former united Andhra Pradesh, Telangana has emerged as the third major state for sweet orange production, after Andhra Pradesh and Maharashtra.

The cultivation of sweet orange faces numerous challenges from both biotic and abiotic factors, including nutritional disorders, insect pests, and diseases. Significant constraints such as greening, root rot, gummosis, and post-harvest rots have resulted in substantial economic losses. Additionally, various leaf and fruit spot diseases have become increasingly prevalent in recent years, leading to severe pre-harvest fruit drop (Sangle *et al.*, 2023). As part of our efforts to identify new and emerging diseases, we are mandated to conduct annual surveys of sweet orange orchards in the major growing districts of Andhra Pradesh.

2. Materials and Methods

2.1. Sampling and pathogen isolation

In July- August of 2023, plants exhibiting symptoms of dark brown, circular or irregular spots on the leaves and fruits of sweet orange trees were collected at CRS, Tirupati. Initially, the lesions on the leaves were confined to specific veins; however, they eventually spread and coalesced across multiple veins. In cases of severe infection, entire symptomatic leaves were found to be dead. The symptoms observed on mature fruits ranged from small specks to large pockmarks, with later stages of infection sometimes resulting in corky tissue erupting from the surface, which could eventually fall out, creating crater-like formations. In immature fruits, symptoms presented as slightly depressed brown to black lesions encircled by yellow halo areas. During my observations, leaf and fruit samples displaying these symptoms were collected. For further analysis, small square sections (5 mm^2) were excised from the margins of the lesions using a sterile scalpel. These samples were then surface sterilized with 70% ethanol for one minute, followed by three rinses with sterile distilled water, and subsequently placed on PDA medium in sterile petri plates. The inoculated plates were incubated at 25°C under a 12-hour photoperiod for seven days. After the incubation period, we noted the development of brownish, ashy, woolly mycelia on the medium, which later changed to a blackish-grey colour. The isolated pathogen was then purified using a single spore isolation technique.

2.2. Morphological Characteristics

From the purified culture, conidia were observed under a compound microscope. The colony morphology, including colour and shape, as well as the characteristics of the conidiophores and conidia, were examined. Colour, shape, and size of at least 25 conidia were recorded, followed by sub-culturing the pathogen through single spore isolation. Pathogenicity tests and molecular characterization were subsequently conducted.

2.3. Pathogenicity Tests

The pathogenicity test was performed using the detached leaf and fruit assay technique. Freshly harvested citrus fruits and leaves, which were free from defects or injuries and uniform in size, colour, and ripeness, were collected for this purpose. The selected sweet orange fruits and leaves were randomized, surface sterilized with 1% sodium hypochlorite, and thoroughly washed under running tap water. After air drying, the samples were wounded and maintained at room temperature for 30 minutes. Each fruit was punctured with a sterile nail (33 mm) at two equidistant points on the equatorial surface, while the leaves were pricked on the abaxial side using three entomological pins mounted on a cork. For each isolate (leaf and fruit), conidial production was induced as outlined by Shahin and Shepard (1979). Actively growing mycelium from the edges of 5-day-old PDA cultures was cut into 1 cm squares, with any fluffy aerial mycelium removed using a sterile scalpel. These squares were then placed on water agar supplemented with calcium carbonate, and 2 ml of sterile distilled water was added on top of the plugs. The Petri plates were incubated in the dark at 20°C, and spores from the square plugs and water agar were harvested after 24-48 hours by filtering through four layers of sterile cheesecloth. The conidial suspension of each isolate was applied to the wounds of 10 fruits and 15 leaves using a sterile spatula, and the samples were then randomly divided into three replicates. The samples were incubated at 24°C with high relative humidity for 10 days. The disease incidence (percentage of infected wounds) and severity (lesion diameter in mm) were recorded. The assay was repeated twice, and Koch's postulates were confirmed by re-isolating the pathogen and comparing it with the original culture.

2.4. Molecular characterization and phylogenetic analysis

The molecular analysis of the culture was conducted to verify the species-level identification. A representative fungal isolate was obtained from each observed symptom and subsequently cultured on Potato Dextrose Agar (PDA)

for a duration of 7 days at room temperature (20 °C). The mycelium from each isolate was carefully collected using a sterile scalpel, and genomic DNA was extracted utilizing the Wizard® Genomic DNA Purification Kit,following the manufacturer's instructions. The internal transcribed spacer (ITS) region of ribosomal DNA was specifically targeted for PCR amplification and sequencing, employing the ITS5 and ITS4 primers.

In addition to the ITS primers, the translation elongation factor 1-α (EF-1α) genes were chosen for their high informative value in differentiating *A.alternata* from other species within the Alternaria section. The primers designated for these regions were EF1-728F and EF1-986R for EF-1α. The PCR mixtures for both ITS and EF-1α included 1 µL of genomic DNA, 2 µM MgCl2, 40 µM of each dNTP, 0.2 µM of each primer, and 0.5 Unit of GoTaq® Flexi DNA polymerase, resulting in a total volume of 12.5 µL. The PCR protocol consisted of an initial denaturation step at 94 °C for 5 minutes, followed by 35 cycles of amplification at 94 °C for 30 seconds, annealing at 48 °C (for ITS) or 52 °C (for EF-1α) for 50 seconds, and extension at 72 °C for 2 minutes, concluding with a final extension at 72 °C for 7 minutes. The resulting PCR products were sequenced in both directions by Eurofins Genomics India pvt.ltd., Bangalore.

The PCR amplifications were executed using a GeneAmp PCR System 9700 (Applied Biosystems). The PCR mixtures for ITS and EF-1α were composed of 1 µL of genomic DNA, 2 µM MgCl2, 40 µM of each dNTP, 0.2 µM of each primer, and 0.5 Unit of GoTaq® Flexi DNA polymerase (Promega), totalling 12.5 µL.

3. Results and Discussion

3.1. Morphological characterization

Fungal cultures derived from infected leaf and fruit samples of sweet orange were purified through the single spore isolation technique. Initially, the culture exhibited a dull white coloration, which subsequently transitioned too ashy grey and eventually black. The presence of black crystals beneath the mycelium mat in the agar medium was also noted (Pryor and Michailides, 2002; Abbasi *et al*., 2018). Following a growth period of 21 days, the cultures were placed in darkness for an additional 3-4 days to promote sporulation. The resulting colonies displayed dark, smooth, septate, and branched conidiophores. The conidia were characterized by cross and longitudinal septa, maintaining a long-elliptical to ovoid-ellipsoidal shape, with 3-6 transverse septa and 1-3 longitudinal septa, exhibiting a pale brown hue and measuring 18.9-36.7 µm

in length and 9.8-11.5 µm in width. These morphological traits were consistent with the genus *Alternaria alternata*, as identified using a taxonomic key (Barnett and Hunter, 1972) (Fr.) Keissl., along with corroboration from other researchers (Abbasi *et al*., 2018). Since 2013, Alternaria disease has been reported in Italy affecting new clones of sweet orange and lemon during the pre-harvest phase. Isolations from diseased leaves and fruits collected from these hosts, as well as from other known Alternaria hosts (*Citrus x clementina, Citrus reticulata* 'Mandalate', and *Citrus sinensis* 'Valencia'), confirmed *Alternaria alternata* as the causative agent (Aiello *et al*., 2020).

Figure 1: Illustrating the symptomatology on Leaves and Fruits of sathgudi sweet orange

3.2. Pathogenicity Tests

The pathogenicity of the culture obtained from symptomatic plants was validated through the detached leaf assay technique. Symptoms manifested on the leaves within 4 to 5 days post-inoculation. Initially, small necrotic spots appeared, which subsequently expanded into larger patches. Similar necrotic spots were observed on the fruits, leading to fruit rot. Re-isolation from these patches on PDA medium confirmed that the morphological characteristics were consistent with the original isolate. Control plants that were not inoculated exhibited no disease symptoms and yielded negative results for fungal isolation. The pathogenicity tests were conducted twice to ensure the reliability of the experimental findings. (Camiletti *et al*., 2022)

Figure 2a: Pathogenicity of the pathogen on the leaves (Detached leaf assay)

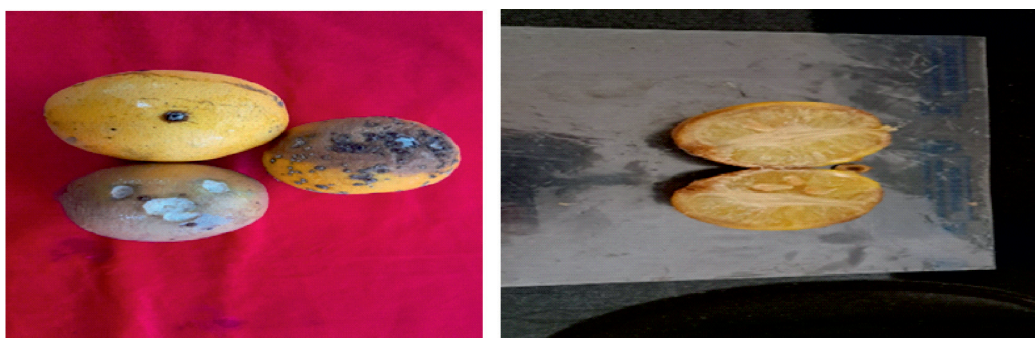

Figure 2b: Pathogenicity of the pathogen on fruit by Pin prick method

3.3. Molecular Characterization

A nucleotide similarity search conducted with type sequences in the NCBI database, utilizing ITS and TEF sequences, indicated a 100 percent similarity with *Alternaria alternata*. Phylogenetic analysis of the nucleotide sequence, performed using the maximum parsimony method in MEGA software, confirmed

the identity of the pathogen as *A.alternata*. The sequence was submitted to GenBank, resulting in the acquisition of the NCBI accession number (PP697737). Molecular detection, carried out using ITS primers, was further corroborated by employing the TEF gene, which received the accession number OM522515, also showing 100 percent similarity with *A.alternata*. Recent reports of Alternaria pathogens causing leaf spots and blight symptoms on sweet orange and other citrus species have emerged from Taiwan (Ni *et al*., 2015), China (Wang *et al*., 2010), Brazil (Chitolina *et al*., 2019), and Pakistan (Moosa *et al*., 2019).

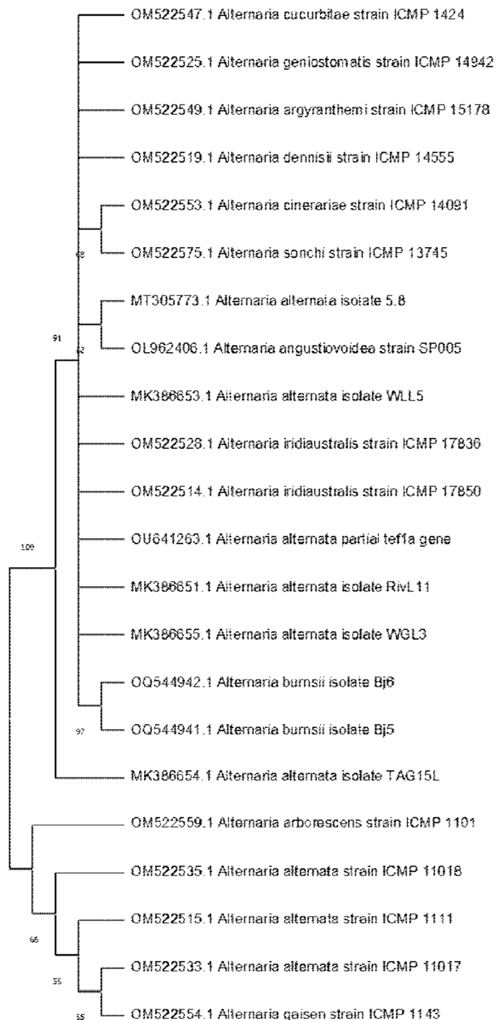

Figure 3: Phylogenetic relationship of *Alternaria alternata* isolated from sweet orange with 20 other Alternaria species which are retrived from NCBI genebank database with TEF-1 region of r DNA

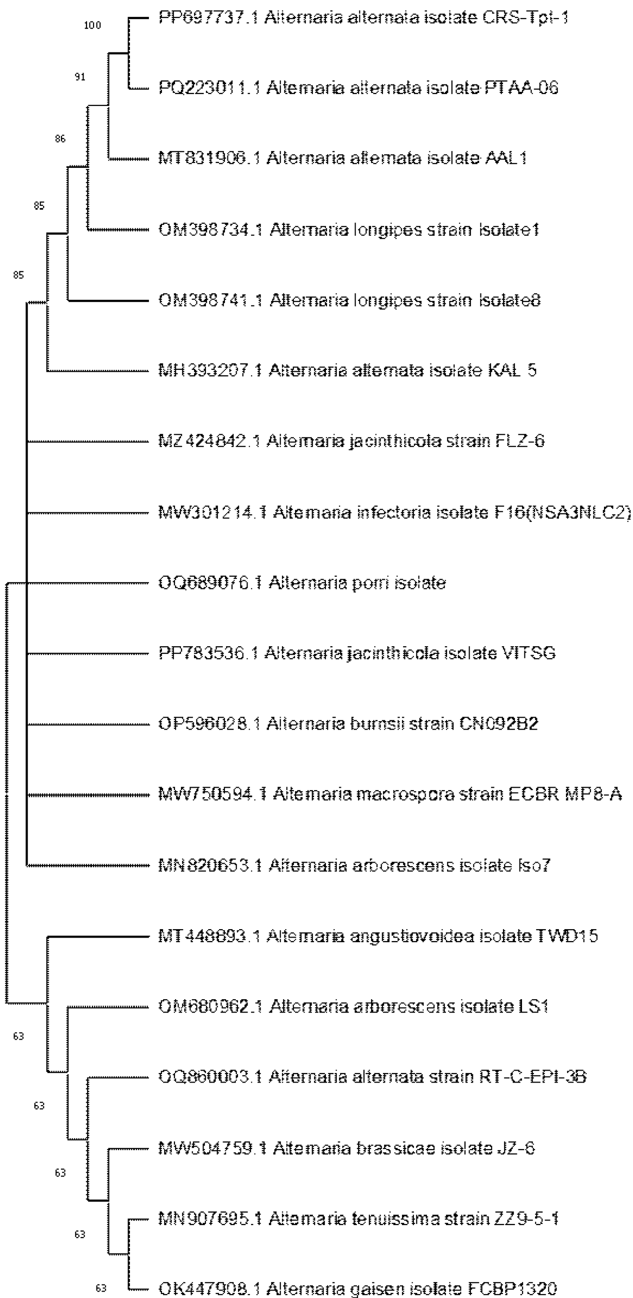

Figure 4: Phylogenetic relationship of *Alternaria alternata* isolated from sweet orange with 20 other Alternaria species which are retrived from NCBI genebank database with ITS region of r DNA

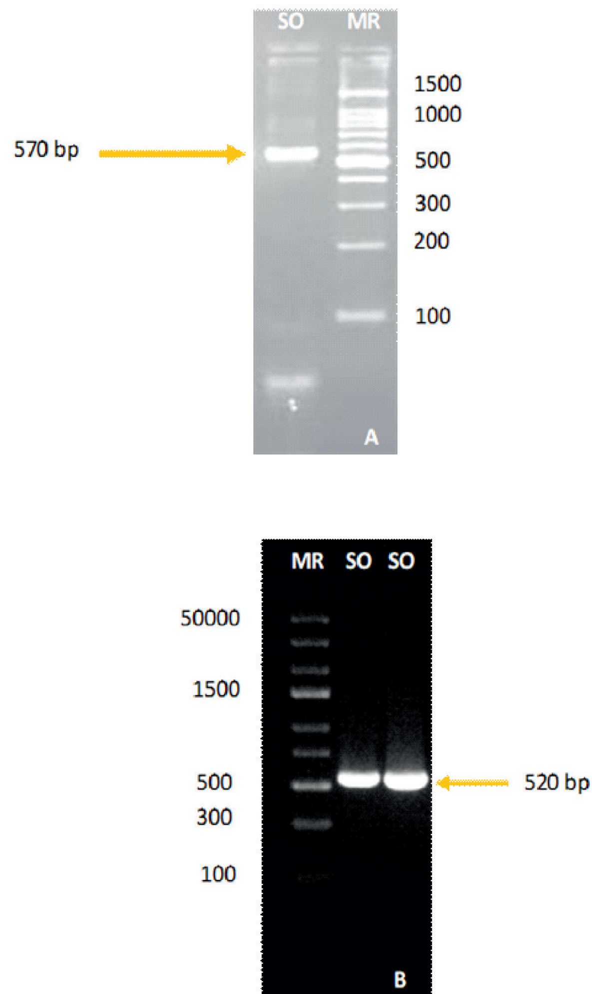

Figure 5: Molecular identification of Alternaria alternata: (A) PCR amplification of the ITS region and (B) PCR amplification of the TEF-1α

4. Conclusion

This study represents the first documented instance of *A.alternata* causing leaf and fruit spot disease in sweet orange within Andhra Pradesh. A phylogenetic analysis utilizing two genes, specifically ITS and TEF sequences from both the pathogen in this study and reference CBS strains, was conducted to accurately identify the isolates. The results confirmed the association of the pathogen with *A.alternata*. While there is considerable existing knowledge regarding citrus Alternaria leaf and fruit spot, further research is essential to investigate the various epidemiological and edaphic factors that influence the pathogen's

Table 1: Similarity matrix of leaf and fruit spot disease in Sweet orange cv. Sathgudi caused by *Alternaria alternate*

Similarity matrix of leaf and fruit spot disease in Sweet orange cv. Sathgudi caused by *Alternaria alternata*

	OM522535.1	OM522515.1	OM522533.1	OM522554.1	OM522559.1	OU641263.1	OM522528.1	MT305773.1	MK386653.1	MK386651.1	MK386655.1	MK386654.1	OM522553.1	OQ544942.1	OM522547.1	OM522525.1	OQ544941.1	OL962406.1	OM522549.1	OM522575.1	OM522514.1	OM522519.1
OM522535.1	ID																					
OM522515.1	1	ID																				
OM522533.1	1	1	ID																			
OM522554.1	0.993	0.993	0.993	ID																		
OM522559.1	0.991	0.991	0.991	0.984	ID																	
OU641263.1	0.767	0.767	0.767	0.764	0.759	ID																
OM522528.1	0.482	0.482	0.482	0.478	0.477	0.427	ID															
MT305773.1	0.795	0.795	0.795	0.788	0.804	0.717	0.461	ID														
MK386653.1	0.759	0.759	0.759	0.756	0.751	0.945	0.409	0.707	ID													
MK386651.1	0.758	0.758	0.758	0.755	0.75	0.947	0.409	0.706	0.997	ID												
MK386655.1	0.759	0.759	0.759	0.756	0.751	0.945	0.409	0.707	1	0.997	ID											
MK386654.1	0.753	0.753	0.753	0.75	0.748	0.937	0.405	0.705	0.987	0.985	0.987	ID										
OM522553.1	0.68	0.68	0.68	0.675	0.677	0.525	0.461	0.586	0.5	0.5	0.5	0.501	ID									
OQ544942.1	0.289	0.289	0.289	0.284	0.287	0.254	0.172	0.346	0.238	0.238	0.238	0.24	0.251	ID								
OM522547.1	0.84	0.84	0.84	0.835	0.839	0.625	0.413	0.697	0.615	0.614	0.615	0.617	0.68	0.282	ID							
OM522525.1	0.855	0.855	0.855	0.849	0.85	0.639	0.419	0.694	0.629	0.628	0.629	0.627	0.689	0.288	0.9	ID						
OQ544941.1	0.289	0.289	0.289	0.285	0.288	0.256	0.173	0.348	0.24	0.239	0.24	0.242	0.248	0.962	0.28	0.287	ID					
OL962406.1	0.634	0.634	0.634	0.629	0.641	0.484	0.357	0.712	0.47	0.469	0.47	0.468	0.494	0.329	0.588	0.587	0.326	ID				
OM522549.1	0.841	0.841	0.841	0.834	0.841	0.621	0.415	0.69	0.612	0.611	0.612	0.61	0.678	0.287	0.912	0.892	0.286	0.581	ID			
OM522575.1	0.647	0.647	0.647	0.643	0.647	0.494	0.445	0.573	0.469	0.47	0.469	0.469	0.86	0.267	0.658	0.659	0.265	0.528	0.65	ID		
OM522514.1	0.603	0.603	0.603	0.597	0.596	0.484	0.361	0.702	0.469	0.468	0.469	0.467	0.467	0.331	0.552	0.554	0.328	0.924	0.543	0.494	ID	
OM522519.1	0.655	0.655	0.655	0.651	0.653	0.51	0.48	0.547	0.487	0.489	0.487	0.486	0.783	0.224	0.644	0.637	0.224	0.45	0.647	0.759	0.422	ID

	PP697737.1	PQ223011.1	MT831906.1	OM398734.1	OM398741.1	MH393207.1	MT448893.1	MZ424842.1	MW301214.1	OQ689076.1	PP783536.1	OP596028.1	OM680962.1	MW750594.1	OQ860003.1	MW504759.1	MN907695.1	OK447908.1	MN820653.1
PP697737.1	ID																		
PQ223011.1	1	ID																	
MT831906.1	0.986	0.986	ID																
OM398734.1	0.869	0.869	0.866	ID															
OM398741.1	0.869	0.869	0.866	1	ID														
MH393207.1	0.892	0.892	0.889	0.944	0.944	ID													
MT448893.1	0.983	0.983	0.975	0.877	0.877	0.899	ID												
MZ424842.1	0.802	0.802	0.799	0.911	0.911	0.871	0.811	ID											
MW301214.1	0.814	0.814	0.811	0.925	0.925	0.885	0.821	0.944	ID										
OQ689076.1	0.875	0.875	0.872	0.973	0.973	0.953	0.882	0.902	0.914	ID									
PP783536.1	0.97	0.97	0.966	0.895	0.895	0.917	0.981	0.828	0.837	0.9	ID								
OP596028.1	0.606	0.606	0.603	0.692	0.692	0.658	0.61	0.717	0.75	0.683	0.625	ID							
OM680962.1	0.972	0.972	0.981	0.858	0.858	0.881	0.968	0.792	0.804	0.864	0.957	0.598	ID						
MW750594.1	0.862	0.862	0.859	0.977	0.977	0.935	0.869	0.92	0.927	0.966	0.886	0.692	0.851	ID					
OQ860003.1	0.959	0.959	0.968	0.848	0.848	0.871	0.955	0.783	0.795	0.855	0.946	0.591	0.985	0.842	ID				
MW504759.1	0.877	0.877	0.874	0.97	0.97	0.953	0.891	0.903	0.906	0.975	0.905	0.676	0.869	0.966	0.86	ID			
MN907695.1	0.864	0.864	0.861	0.977	0.977	0.94	0.877	0.913	0.918	0.966	0.891	0.685	0.856	0.975	0.847	0.979	ID		
OK447908.1	0.884	0.884	0.88	0.964	0.964	0.96	0.898	0.896	0.902	0.97	0.912	0.674	0.876	0.961	0.866	0.992	0.972	ID	
MN820653.1	0.805	0.805	0.802	0.902	0.902	0.873	0.815	0.953	0.951	0.902	0.831	0.723	0.795	0.911	0.786	0.899	0.902	0.896	ID

survival and dissemination across different agro-ecological zones in Andhra Pradesh. Additionally, it is important to explore a range of cultural, biological, and chemical management strategies to effectively address this disease.

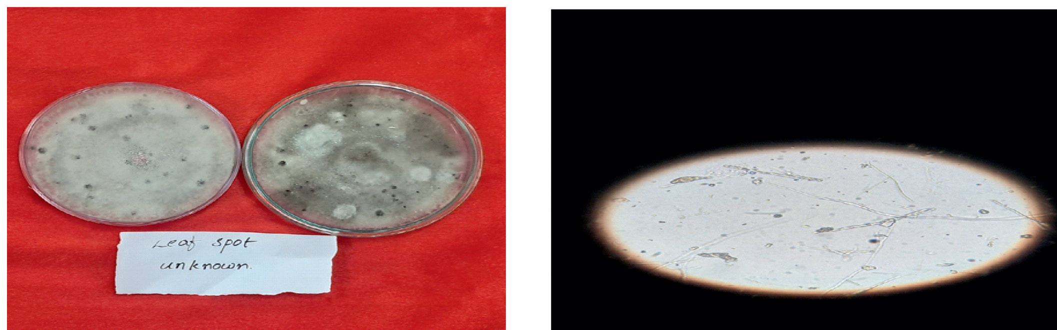

Figure 6a : Culture of the pathogen isolated from the symptomatic plant
6b: Conidia of the isolated pathogen

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