

RESEARCH ARTICLE

MOLECULAR AND PATHOLOGICAL EVIDENCE OF *Colletotrichum fructicola* INFECTING ONION (*Allium cepa* L.) IN SRI LANKA

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ABSTRACT

Anthracnose–twister disease is a significant fungal threat to onion (*Allium cepa* L.) production in Sri Lanka, particularly in dry and intermediate zones. Although species such as *Colletotrichum gloeosporioides* and *C. siamense* have been previously implicated, the full pathogen spectrum remains unresolved. During the *Maha* season (2022–2023), symptomatic onion plants were surveyed across major cultivation areas, and isolates were obtained from diseased tissues. Morphological characterization revealed colonies with greyish-white aerial mycelia and orange to salmon-colored acervuli, while microscopic features were consistent with *Colletotrichum* spp. Molecular identification based on multilocus phylogenetic analysis (ITS, actin, and histone H3 genes) confirmed the identity of the isolates as *Colletotrichum fructicola*. Pathogenicity assays reproduced field symptoms within 7–8 days post-inoculation and successfully fulfilled Koch's postulates. This study presents the first report of *C. fructicola* as a causal agent of anthracnose–twister disease in onion in Sri Lanka, expanding the known diversity of the *C. gloeosporioides* species complex associated with this crop. The findings underscore the importance of molecular diagnostics in disease surveillance and have practical implications for integrated disease management strategies in tropical onion production systems.

Keywords: *Allium cepa*, anthracnose-twister, *Colletotrichum fructicola*, pathogenicity, phylogenetics

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1. INTRODUCTION

Onion (*Allium cepa* L.) is one of the widely cultivated vegetable crops in Sri Lanka, contributing significantly to both the national rural economy and food security. It is predominantly grown in the dry and intermediate zones during the *Maha* (wet) and *Yala* (dry) seasons [1]. However, onion production is often hindered by biotic stresses, particularly fungal diseases such as purple blotch, Stemphylium blight, downy mildew, and anthracnose-twister disease, all of which can lead to substantial losses in yield and quality [2 - 4].

Anthracnose-twister disease is characterized by leaf curling, twisting, chlorosis, abnormal elongation of the pseudostem, and the development of sunken lesions on lower leaves. These lesions often bear pink masses of conidia and may progress to bulb rot [2]. The disease spreads rapidly under favorable climatic conditions, particularly high relative humidity (85–96%), moderate temperatures (20–31 °C), and frequent rainfall [5,6].

Early studies in Sri Lanka, based on morphological identification, implicated *Colletotrichum gloeosporioides* and *Fusarium oxysporum* as the main pathogens associated with the anthracnose-twister complex [2,3]. Recently, *C. siamense* was reported from onion fields in Sri Lanka [7]. Several *Colletotrichum* species have been reported as pathogens of onion in various countries, highlighting the taxonomic complexity and host adaptability of the genus. In Brazil, Lopes *et al*. [8] identified five anthracnose-causing *Colletotrichum* species: three belonging to the *C. acutatum* species complex (*C. nymphaeae*, *C. scovillei*, and *C. tamarilloi*), and two from the *C. gloeosporioides* complex (*C. fructicola* and *C. theobromicola*). Similarly, in Taiwan, Yu *et al*. [9] reported the presence of *C. spaethianum* and *C. circinans* as causative agents of onion anthracnose. These findings underscore the global diversity of *Colletotrichum* species affecting onion and emphasize the need for region-specific pathogen surveillance.

Accurate identification of *Colletotrichum* species associated with anthracnose-twister disease remains challenging due to the morphological similarity and cryptic nature of many taxa within the *C. gloeosporioides* species complex. Molecular approaches, particularly multilocus phylogenetic analyses of conserved genes, have proven effective in resolving species boundaries and have revealed a wider diversity of *Colletotrichum* species causing anthracnose in various crops worldwide [10, 11].

This study focuses on a fungal species associated with onion anthracnose–twister disease that has not been previously reported in Sri Lanka. The pathogen was isolated and confirmed through detailed morphological characterization, molecular identification, and pathogenicity testing. This first report provides a scientific basis for further studies on the diversity, epidemiology, and management of anthracnose pathogens affecting onion in the country.

2. MATERIAL AND METHODS

2.1. Study Area and Disease Survey

Field surveys were conducted during the *Maha* cropping season (December 2022 to February 2023) across three onion-growing districts of Sri Lanka: Kilinochchi, Mullaitivu, and Anuradhapura. To assess the prevalence of anthracnose-twister disease, 15 onion fields were randomly selected in each district. For detailed evaluation of disease incidence (DI) and disease severity (DS), five farms were randomly chosen from each district. Within each selected field, ten sampling sites were identified using a randomized approach. Each site comprised 30 onion plants, providing a representative sample for disease assessment. The prevalence, DI, and DS were calculated using standard phytopathological formulas to ensure consistency and comparability across sites. Prevalence % = (Number of locations showing symptomatic plants)/ (Total number of locations per district) X 100. DI (%) = (Number of infected plants/Total number of plants observed) x 100. DS was measured using the methodology described by Albert *et al*. [12]. DS % = (Σ (v X n))/ (Z X N) X 100%, where; v = scale value of each category of attack, n = number of plants per category of attack, Z = scale value of high attack category, and N = number of plants observed. Statistical analyses were performed using SPSS software version 26.0. An analysis of variance (ANOVA) was conducted to assess significant differences in disease incidence and severity among the three surveyed districts. Where significant effects were detected, Tukey's Honest Significant Difference (HSD) test was used for post hoc pairwise comparisons. All statistical tests were conducted at a 5% significance level ($p<0.05$).

2.2. Disease Sample Collection and Fungal Isolation

Onion plants exhibiting characteristic symptoms of anthracnose-twister disease were collected from the surveyed districts. Symptomatic samples were placed in sterile polyethylene bags and transported to the laboratory for processing within 12–24 hours to preserve pathogen viability. Infected tissues were excised into small segments and surface sterilized using 1% sodium hypochlorite (NaOCl) for 1 min, followed by three successive rinses in sterile distilled water. The sterilized tissue segments were then plated onto Potato Dextrose Agar (PDA) and incubated at 27 ± 2 °C. Emerging fungal hyphae were subcultured onto fresh PDA plates to obtain pure cultures for further analysis.

2.3. Morphological Characterization

For morphological and cultural characterization, mycelial discs (4 mm in diameter) from actively growing pure fungal cultures (M3, K3, and A1) were aseptically transferred to the center of PDA plates and incubated at 27 ± 2 °C for 10 days. Colony diameter was measured to determine radial growth, and qualitative observations were made on colony morphology, including texture, pigmentation (both upper and lower surfaces), margin characteristics, and growth patterns. Microscopic features, including conidial morphology and hyphal characteristics, were examined by mounting fungal material in lactophenol cotton blue and observing under a compound microscope (Olympus CX31, Japan). Spore dimensions were measured from 30 randomly selected conidia using a calibrated ocular micrometer.

2.4. Molecular Characterization and Phylogenetic Analysis

Genomic DNA was extracted from three representative fungal isolates (A1, M3, and K3) using a commercial kit (Wizard® Genomic DNA Purification Kit, Promega). Polymerase Chain Reaction (PCR) amplification targeted partial sequences of three genomic regions: internal transcribed spacer (ITS) region (primers ITS1 /ITS4) [13], the actin (*act*) gene (primers ACT512F/ACT783R) [14], and the histone H3 (*his3*) gene (primers CYLH3F/CYLH3R) [15]. Amplicons were sent to Macrogen (Seoul, Republic of Korea) for bidirectional Sanger sequencing. The resulting sequences were analyzed using BLASTn against the NCBI GenBank database to confirm species-level identity. Sequences were deposited in GenBank under the following accession numbers: *his3*-

PV565072 (A1), PV550995 (M3), PV550994 (K3); *act*- PV882375 (A1), PV565030 (M3), PV565029 (K3); and ITS- PV471379 (A1), PV163124 (M3), PV163116 (K3).

For phylogenetic analysis, the ITS, *act*, and *his3* gene sequences of representative *Colletotrichum* ex-type strains were retrieved from GenBank. Multiple sequence alignments were performed using MUSCLE. Maximum Likelihood (ML) analysis was performed in MEGA12 (version 12.0.13) employing the Tamura-Nei model with 1,000 bootstrap replications. *Monilochaetes infuscans* strain CBS 869.96 was used as the outgroup. Additionally, Bayesian analysis was conducted in BEAST version 10.5.0, with four independent runs of 10,000,000 iterations each, sampling every 1,000 iterations, using the HKY substitution model and Yule speciation prior. The resulting log files were analyzed in Tracer version 1.7.2 to assess convergence and effective sample sizes. The first 10% of trees were discarded as burn-in, and the remaining trees were combined using Log Combiner version 10.5.0. Posterior probabilities (PP) were estimated with Tree Annotator version 10.5.0.

2.5. Pathogenicity Confirmation

Pathogenicity assays were performed under greenhouse conditions to fulfill Koch's postulates. Healthy onion bulbs were planted in sterilized pots and maintained until the three to four-leaf stage. Three isolates, namely, A1, K3, and M3, previously obtained from symptomatic field samples, were used for inoculation. Conidial suspensions were prepared from 10-day-old cultures of each isolate and adjusted to a concentration of 1×10^6 conidia ml^{-1}. The suspensions were sprayed onto the foliage of five replicate onion plants per isolate. Control plants were treated with sterile distilled water. All plants were maintained under high-humidity conditions at 27 ± 2 ºC and monitored daily for the development of disease symptoms. Following symptom manifestation, pathogens were successfully re-isolated from infected tissues. These re-isolates were compared morphologically with the original field isolates to confirm their identity, following the procedures described by Than *et al.* [16].

3. RESULTS

The affected onion plants exhibited typical symptoms of anthracnose–twister complex on both leaves and bulbs (Fig.1). Early symptoms included the appearance of small white spots on leaf surfaces, which gradually expanded into chlorotic, concentric rings. These lesions coalesced over time, covering extensive portions of the foliage. As the disease progressed, acervuli developed in concentric patterns within the necrotic tissues, often accompanied by prominent salmon-coloured conidial masses. Twister symptoms were also evident, including leaf curling, abnormal elongation of the neck, and slight twisting of foliage. In advanced stages, leaves became slender, chlorotic, and eventually underwent defoliation, dieback, and plant collapse. Salmon-coloured conidial masses were additionally observed on the outer bulb scales, resulting in bulb deformation. In severely infected plants, complete wilting and death were recorded.

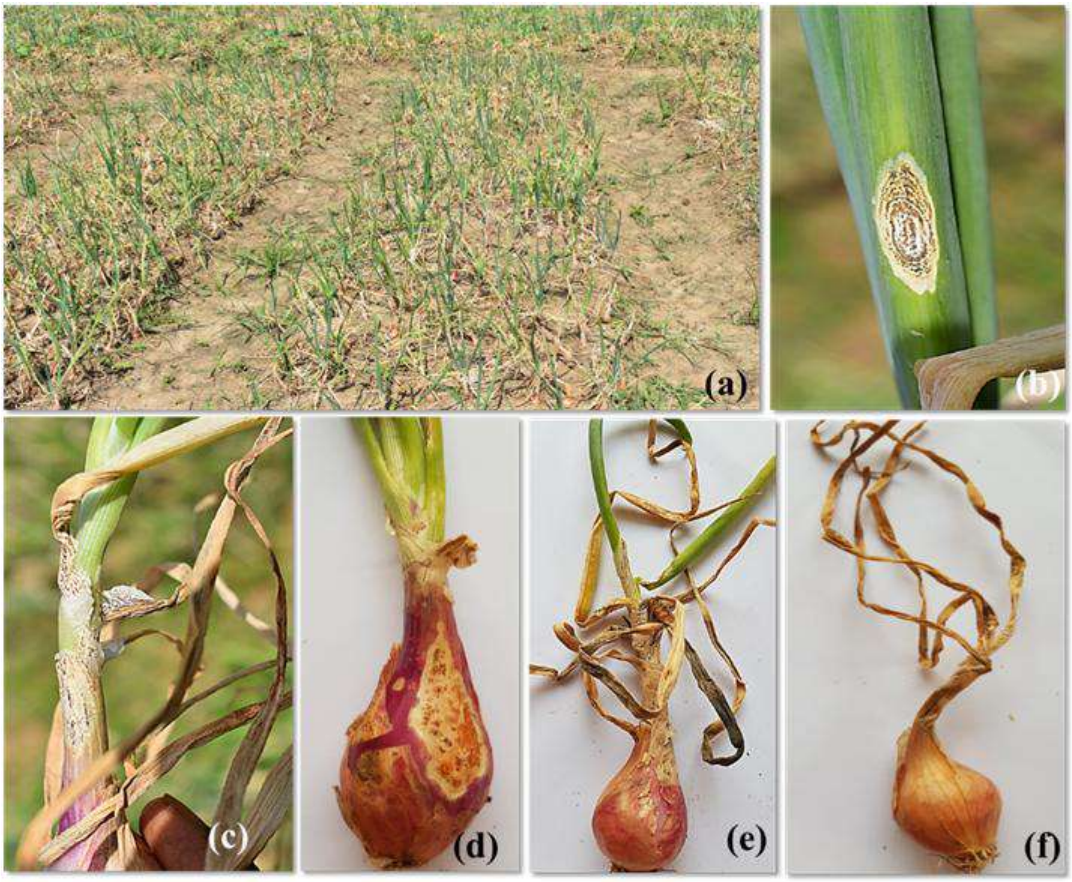

Figure 1 - Symptoms of anthracnose-twister disease in onion (a) Field view of infection. (b) Necrotic lesion with concentric rings. (c) Twisting and curling (d) Infected onion bulb (e) Infected onion plant (f) Dead plant

Field surveys revealed significant variation in DI and DS of anthracnose–twister disease among the surveyed districts (DI: F (2,12) = 364.52, p < 0.001; DS: F (2,12) = 302.49, p < 0.001). The DI and DS were higher in Anuradhapura (80.4% and 67.3%, respectively) compared with the other districts. Kilinochchi showed moderate disease levels (35.8% incidence, 19.6% severity), whereas Mullaitivu exhibited the lowest values (30.1% incidence, 13.6% severity).

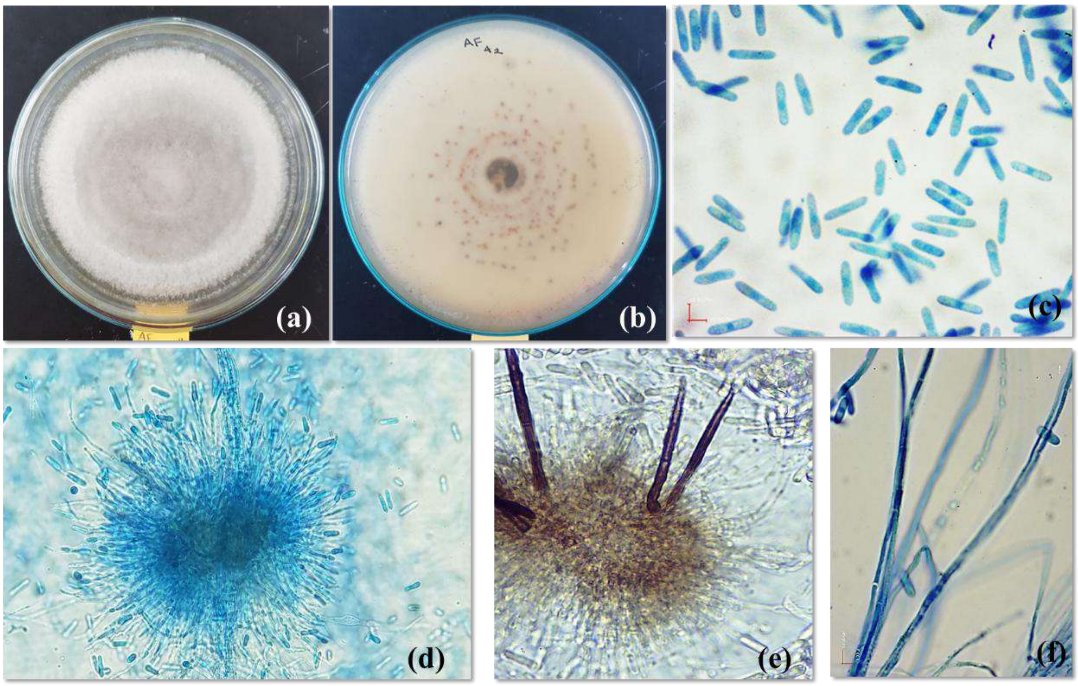

Figure 2 - Cultural and morphological characteristics of Colletotrichum fructicola isolate (A1) associated with anthracnose-twister disease in onion. ***(a)*** *upper surface and* ***(b)*** *reverse side of the culture on PDA,* ***(c)*** *conidia,* ***(d)*** *conidiophores and conidial masses.* ***(e)*** *setae* ***(f)*** *hyaline mycelia.*

Fungal isolates from diseased onion samples exhibited a mean radial growth rate of 7.8 mm per day on PDA. The colonies produced cottony, greyish-white aerial mycelia, with distinct orange to salmon-coloured concentric acervuli. Microscopic examination revealed unicellular, hyaline, aseptate, smooth-walled conidia, which were cylindrical with rounded apices, measuring 10.11–13.82 × 2.23–2.97 μm (n = 90). Conidiophores were hyaline and cylindrical to subcylindrical in shape. Based on these morphological features, the isolates were identified as belonging to the genus *Colletotrichum*, consistent with descriptions provided by Lopes *et al*. [8] and Weir *et al*. [10].

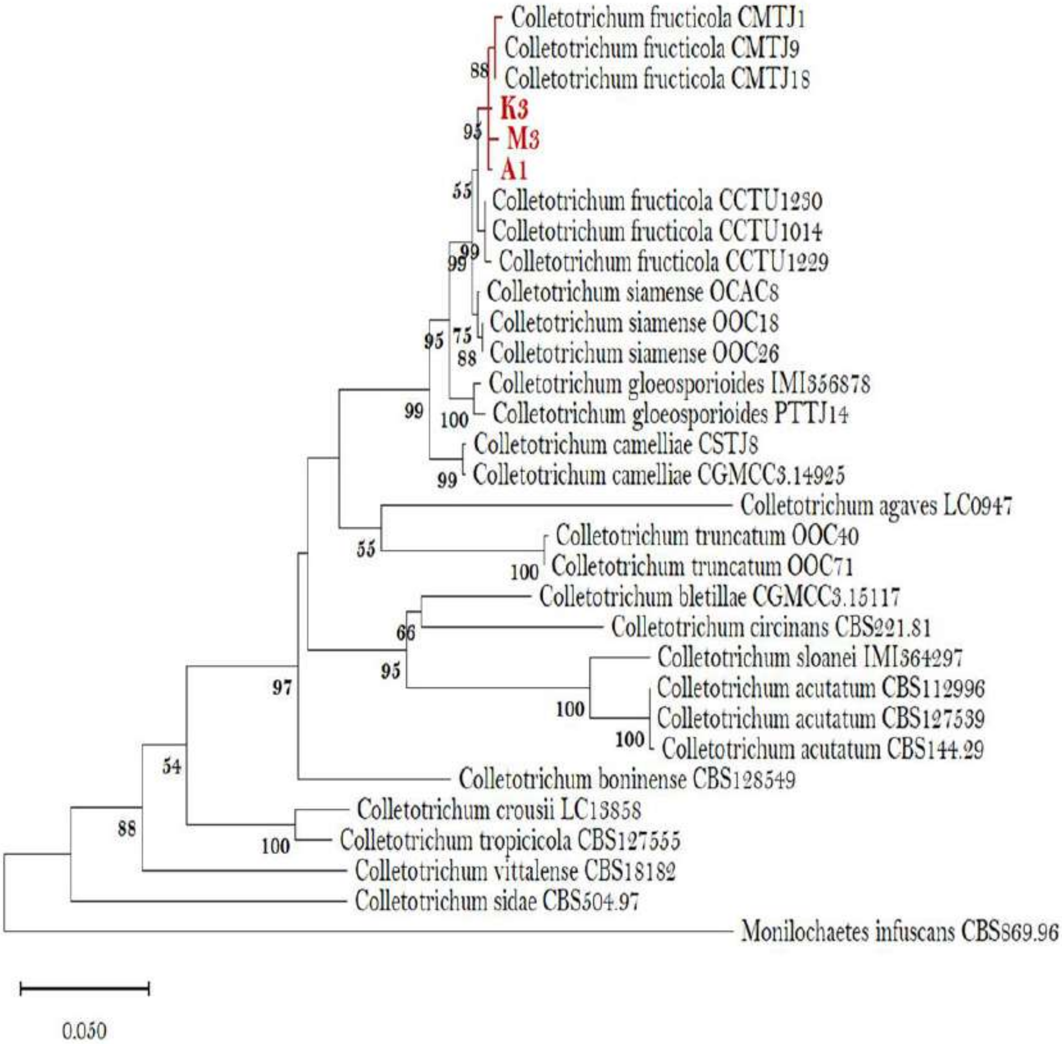

Figure 3a - Phylogenetic tree based on concatenated ITS, act, and his-3 gene sequences showing the placement of Colletotrichum fructicola isolates (A1, M3, K3; highlighted in red). The tree was constructed using the Maximum Likelihood method (Tamura-Nei-T93+G+I model). Bootstrap values (percentages) greater than 50% from 1000 replicates are indicated at the nodes. Monilochaetes infuscans CBS869.96 was used as the outgroup.

Multilocus phylogenetic analysis using ITS, *act*, and *his3* gene regions was conducted to determine the species identity of fungal isolates A1, M3, and K3. The ML tree (Fig. 3a) grouped the three isolates into a strongly supported monophyletic clade with known strains of *C. fructicola*, with a bootstrap value of 95%. Similarly, the Bayesian inference tree (Fig. 3b) showed consistent topology, placing the isolates within the *C. fructicola* clade with a PP of 1.0. In both analyses, the isolates were clearly separated from the other closely related species, such as *C. siamense* and *C. gloeosporioides*, which clustered in distinct and well-supported clades. These results confirm that A1, M3, and K3 share a close evolutionary relationship with reference *C. fructicola* strains and are genetically distinct from the other *Colletotrichum* taxa.

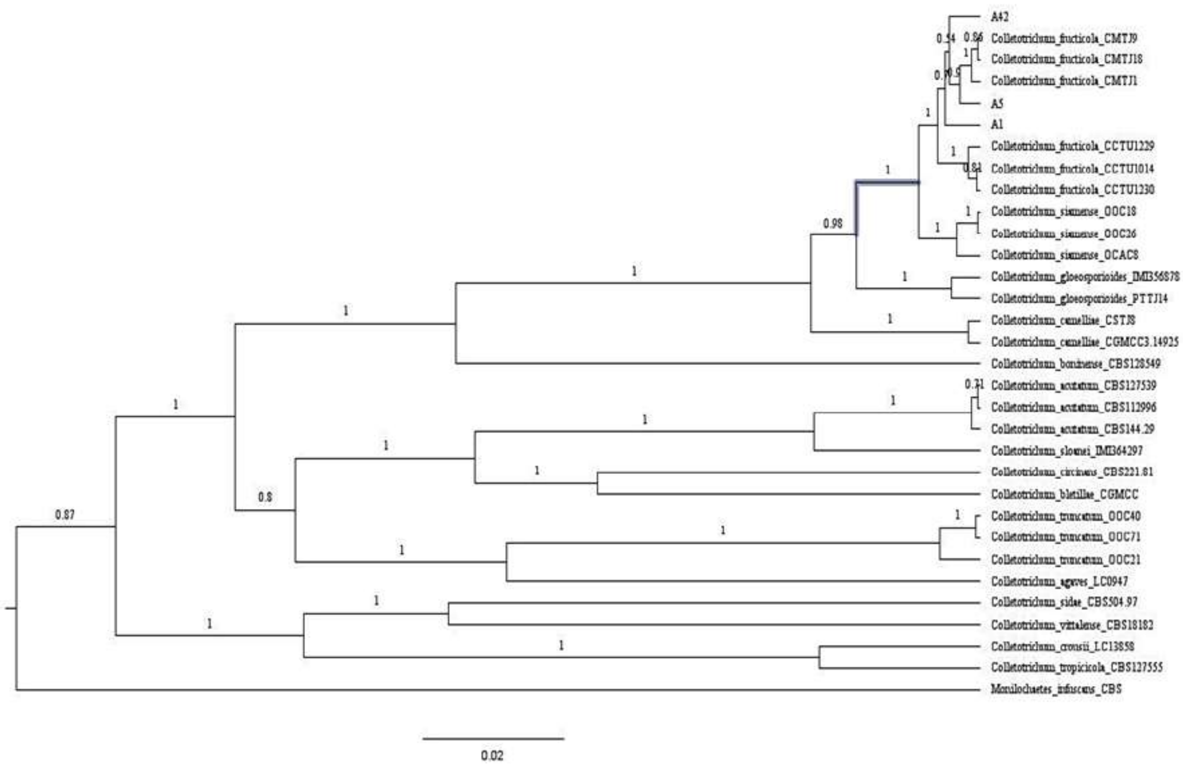

Figure 3b - Bayesian inference phylogenetic tree based on multi-locus sequence data (ITS, act, his3) showing the relationships among Colletotrichum. Posterior probability values are indicated at each node. Isolates from the present study are A1, M3, and K3. Monilochaetes infuscans was used as the outgroup. The scale bar represents 0.02 nucleotide substitutions per site.

Pathogenicity tests confirmed the ability of the fungal isolates to reproduce disease symptoms under controlled conditions. Inoculated onion plants developed typical anthracnose–twister symptoms within 7–8 days post-inoculation (Fig. 4), closely resembling those observed in naturally infected field plants. In contrast, control plants treated with sterile distilled water remained symptomless throughout the observation period.

Figure 4 - Pathogenicity testing of C. fructicola on onion under controlled conditions. (a) Whole plant assay showing healthy control (X) and inoculated plant (Y). (b) Inoculated plant showing typical twisting, necrosis, and drying of leaves.

4. DISCUSSION

This study presents the first molecular and pathological confirmation of *C. fructicola* as a causal agent of anthracnose–twister disease in onion in Sri Lanka. Previous reports from the Anuradhapura and Matale districts have implicated *C. gloeosporioides* and *C. siamense* in similar disease manifestations [2,7]. The identification of *C. fructicola* adds to the known diversity within the *C. gloeosporioides* species complex affecting onion and signifies a potentially emerging pathogen in Sri Lankan agroecosystems.

Morphological and cultural characteristics observed in the present isolates, such as the production of salmon-colored acervuli and cylindrical, aseptate conidia, are consistent with earlier reports of *C. fructicola* [8,10]. Multilocus sequence analysis enabled precise species delimitation. The isolates grouped robustly with reference strains of *C. fructicola*, supported by high bootstrap (95%) and posterior probability (1.0) values. This reflects the versatility of multilocus phylogenetics in distinguishing cryptic species [10,17]. The ability to distinguish *C. fructicola* from *C. siamense* and *C. gloeosporioides* is critical for disease diagnosis and resistance screening, particularly as multiple *Colletotrichum* species may co-exist within the same cropping systems [18].

Pathogenicity assays demonstrated the virulence of the *C. fructicola* isolates, inducing disease symptoms in 7–8 days post-inoculation. These included leaf curling, neck elongation, and eventual plant collapse, symptoms similar to those described in *C. gloeosporioides* infected onions [12,19]. Koch's postulates were fulfilled, thereby confirming causality. Notably, *C. fructicola* has been associated with severe twister symptoms in this study, suggesting a possible role in hormone-mediated physiological disruptions, which warrants further investigation.

Geographical variation in disease incidence, with the highest levels recorded in Anuradhapura, likely reflects a combination of factors beyond basic agro-climatic classification. While all three surveyed districts (Anuradhapura, Kilinochchi, and Mullaitivu) fall within Sri Lanka's Low Country Dry Zone, the observed differences may stem more from agronomic practices, irrigation regimes, and intensity of onion cultivation than from climatic divergence alone. Anuradhapura is a well-established agricultural hub with larger-scale commercial onion production, frequent cropping cycles, and greater irrigation coverage, which may enhance inoculum pressure and create micro-environments favorable to *C. fructicola* development. In contrast, Kilinochchi and Mullaitivu support smaller, more fragmented cultivation areas, potentially limiting pathogen build-up and disease spread. These findings align with previous work by Pangilinan [19] and Patil [20], who emphasized the importance of crop phenology, field humidity, and cultural management in driving anthracnose outbreaks under relatively similar macroclimatic conditions.

Importantly, *C. fructicola*, previously reported in Sri Lanka on anthurium [18], is confirmed here for the first time in onions, expanding its known host range. Its recent emergence in Brazil's onion fields [8] and now across multiple districts in Sri Lanka underscores its epidemiological flexibility and aggressive pathogenic potential, necessitating targeted management and further surveillance across different cropping systems and zones.

4. CONCLUSION

This study adds *C. fructicola* to the list of pathogens associated with anthracnose–twister disease in onions in Sri Lanka. Confirmed through morphological, molecular, and pathogenicity analyses, our findings expand the known diversity of *Colletotrichum* species linked to this disease and underscore the importance of precise identification for effective disease management.

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