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Morpho-molecular characterisation of *Allocanariomyces* spp. causing die-back of chilli

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Abstract
Die-back of chilli (*Capsicum annuum* L.) represents a complex disease associated with multiple pathogenic organisms and emerged as a major constraint to sustainable chilli production. The disease causes recurrent outbreaks leading to substantial yield losses and posing a serious threat to crop productivity. In the present investigation, a random roving survey was conducted across the principal chilli-growing regions of Karnataka to determine intensity of die-back. Symptomatic plant were collected from fields and subjected to pathogen isolation on potato dextrose agar medium under aseptic conditions. The cultural and morphological characterization of the fungal isolate revealed colonies that were greyish green centre with white margin on both the sides of the plate. Conidia were produced singly, typically humicola-like and borne on the tips of hyphae. For molecular confirmation, genomic DNA was extracted from pure cultures and the Internal Transcribed Spacer (ITS) region of ribosomal DNA was amplified using universal primers ITS-1 and ITS-4, resulted in an amplicon of approximately 560 base pairs. The amplified product was sequenced and subjected to BLAST analysis against sequences available in the NCBI GenBank database. The sequence exhibited high similarity with reference sequences of *Allocanariomyces* sp., thereby confirming its identity as the causal organism associated with chilli die-back in the surveyed regions.

Keywords: *Allocanariomyces spp,* chilli, die-back, karnataka, morpho-molecular characterisation

Introduction
Chilli (*Capsicum annuum* L.) a member of the family Solanaceae, is one of the most economically significant spice crops cultivated globally. It is known for its pungency, flavour, colour and diverse nutritional and medicinal properties (Wahyuni *et al.*, 2013) [14]. Chilli is consumed in fresh, dried and processed forms, serving as a key component of culinary practices and a major export commodity, particularly for India. Its economic and nutritional importance arises from its rich profile of bioactive compounds including capsaicin the alkaloid responsible for pungency; carotenoids, which confer characteristic colouration (Nishani *et al.*, 2021) [9] and several vitamins and micronutrients, notably vitamins A and C, dietary fibre and folic acid (Kantar *et al.*, 2016) [6].
Globally, chilli is cultivated over approximately 2.0 million hectares, yielding 38.60 million tonnes of green chilli and 4.60 million tonnes of dry chilli annually (Anon., 2023). India ranks as the world's largest producer and exporter of dried chillies contributing nearly 35–40 per cent of global production and approximately 25 per cent of total exports (Anon., 2023). Within India, chilli occupies an area of 8–9 lakh hectares with an estimated production of 1.70–2.00 million tonnes of dry chilli and 2.20–2.50 million tonnes of green chilli. During 2024–25, Karnataka reported 2.19 lakh hectares under chilli cultivation, producing 3.36 lakh tonnes with an average productivity of 1.53 t ha^{-1} (Anon., 2025).
Among the various biotic constraints affecting chilli production, die-back disease has emerged as a major limiting factor. The disease is typically characterized by necrosis, wilting and progressive die-back of twigs and branches, ultimately leading to partial or complete plant mortality (Strouts and Winter, 2000) [13]. This disease not only reduces yield and fruit quality but also poses a significant threat to the sustainability of chilli cultivation in major growing regions.

Materials and Methods
Survey and collection of samples
A random roving survey was conducted in major chilli growing areas of Raichur, Ballari, Gadag, Yadgir, Dharwad, Koppal and Haveri of Karnataka to collect the diseased samples during 2024-25. Disease severity was calculated by using the formulae given by Wheeler (1969) [15]. The disease scoring was done based on the scale given by Sharma *et al.* (2004) [12]

$$\text{Per cent disease index (PDI)} = \frac{\text{Sum of all ratings}}{\text{Total number of plants scored} \times \text{maximum scale}} \times 100$$

$$\text{Disease incidence (\%)} = \frac{\text{No. of plants infected}}{\text{No. of plants observed}} \times 100$$

Disease rating scale

Score values	Per cent foliage covered
0	Healthy
1	0.1- 5 %
2	6-10 %
3	11-25 %
4	26-50 %
5	Above 50 %

Diseased chilli samples (n=15) exhibiting characteristic die-back symptoms were collected from different villages across

major chilli-growing districts of Karnataka. The infected twigs and stems were cut into small segments along with adjoining healthy tissue. The segments were surface sterilized with 0.1% mercuric chloride ($HgCl_2$) for 30 seconds, followed by three successive rinses with sterile distilled water to remove residual disinfectant. The sterilized tissues were wet dried on filter paper and aseptically placed on potato dextrose agar medium in Petri plates. Each sample was plated in triplicate, with four tissue segments per replicate and incubated at 25 ± 2°C for 5–7 days (Espinoza *et al.*, 2009) [5]. Fungal colonies from the infected tissues were sub-cultured to obtain pure cultures using the hyphal tip method. The purified isolates were maintained on potato dextrose agar slants and stored.

Isolation and morphological characterization

The cultural characteristics such as colony colour, texture, growth pattern and pigmentation were recorded. Morphological features including hyphal colour and septation, conidial shape, size and colour were examined under microscope.

Pathogenicity test

The pathogenicity of the isolated fungus was done by twig inoculation method (Bhat *et al.*, 2017) [4]. Healthy 45-day-old chilli plants grown under greenhouse conditions were used for the assay. The plants were surface-sterilized and a T-shaped incision was made on the shoot tips. Small mycelial discs (3 mm diameter) from 7-day-old pathogen culture of the test isolate were aseptically inserted into the cuts which were then wrapped with parafilm and covered with a transparent polyethylene sheet to maintain humidity and facilitate infection. For control treatments, sterile distilled water were inserted instead of mycelial plugs and each treatment, including the control, was replicated three times. The plants were maintained under controlled conditions and observed daily for symptom development. the severity was calculated by using above mentioned scale (Sharma *et al*., 2004) [12]. The re-isolation of the pathogen from artificially inoculated plants was performed to satisfy Koch's postulates and confirm the causal relationship between the pathogen and disease symptoms.

Molecular characterisation

Genomic DNA was extracted from 7 day-old actively growing mycelium of the representative fungal isolate using the Cetyl Trimethyl Ammonium Bromide (CTAB) method described by Möller *et al.* (1992). The Internal Transcribed Spacer (ITS) region of ribosomal DNA (rDNA) was amplified using the universal primers ITS-1 and ITS-4 in a HI-MEDIA thermocycler. The PCR reaction mixture (25 µL) contained PCR buffer, 1.5 mM $MgCl_2$, 200 µM dNTPs, 10 pmol of each primer, 1 U of Taq DNA polymerase, and 50 ng of template DNA. The amplification was carried out under the following thermal cycling conditions: initial denaturation at 94°C for 4 min; 35 cycles of denaturation at 94°C for 30 s, annealing at 48°C for 1 min, and extension at 72°C for 1 min; followed by a final extension at 72°C for 7 min.

The amplified PCR products were analysed by 1.5% agarose gel electrophoresis and visualized under UV transillumination. The PCR product (~560 bp) was purified and subjected to bidirectional sequencing at Eurofins Genomics India Pvt. Ltd., Bangalore. The resulting ITS sequence was edited and compared with existing fungal sequences in the National Center for Biotechnology Information (NCBI) database using the BLASTn program to determine sequence similarity and taxonomic identity. The confirmed sequence of the representative isolate was deposited in the NCBI GenBank, Maryland, USA, to obtain a unique accession number.

Phylogenetic analysis

To elucidate the phylogenetic relationship of the isolate a dendrogram was constructed using the ITS sequence obtained in the present study along with reference sequences of related taxa retrieved from the NCBI-CBS database. Sequence alignment was performed using the Maximum Neighbour-Joining method implemented in MEGA version 12 software (Raja *et al.*, 2017) [11] with 1,000 bootstrap replications to assess the robustness of the branches. The details of the reference sequences and the representative isolate were depicted in the constructed dendrogram.

Result and discussion

Survey and collection of samples

A random roving survey was conducted in the major chilli-growing areas of Karnataka to assess the prevalence of die-back disease. The affected chilli plants exhibited typical die-back symptoms, which initially appeared as necrosis of twigs. These necrotic lesions progressed from the tip downwards, leading to drying of twigs and ultimately resulting in the death of the entire plant (Fig. 1 A and B). The disease incidence and severity were recorded as 35.00 and 37.80 per cent respectively, indicating a moderate to severe occurrence of die-back in the surveyed area.

Isolation and morphological Characterization

Isolation from symptomatic chilli plant parts collected from Manginahalli village, Shahpur taluk, Yadgiri district, Karnataka, consistently yielded fungal growth on potato dextrose agar medium. The fungal colonies (CDMA-1) exhibited very slow growth *i.e.,* 60 mm even after seven days of inoculation. On potato dextrose agar, the colonies were submerged and flat, with a greyish-green centre and white margin on the obverse side, while the reverse side appeared grey at the centre with a white margin (Fig.1 C and D). Microscopic examination revealed that the mycelium was hyaline and septate, with an average width of 3.78 µm (n = 10). The conidia were single-celled, hyaline, Humicola-like, and produced solitarily on aerial hyphae (Fig. 1 E). The diameter of the conidia ranged from 3.78 µm (Mehrabi *et al.,* 2020) [7]. Based on these cultural and morphological characters, the isolated pathogen was identified as *Allocanariomyces* sp.

Pathogenicity test

Pathogenicity of the fungal isolate CDMA-1 of *Allocanariomyces* was confirmed through artificial inoculation under controlled conditions. Forty-five-day-old chilli plants were inoculated with mycelial bits of the isolate on the tender twigs. Inoculated plants developed typical die-back symptoms identical to those observed under field conditions, characterized by necrosis of twigs progressing from the tip downward, leading to twig drying and die-back and death of plant occurs and the severity was about 80.00 per cent. In contrast, the uninoculated control plants remained completely symptomless throughout the

observation period (Fig. 2). The pathogen was successfully reisolated from the artificially infected plants and its morphological characteristics were found to be identical to the original isolate, thereby fulfilling Koch's postulates. These results clearly depicts *Allocanariomyces* sp. isolate CDMA-1 as the causal organism responsible for die-back in chilli plants and these were in accordance with Pazooki *et al.* (2022) [10].

Molecular characterisation

For further confirmation of the pathogen, molecular characterization was carried out for isolate CDAC-1. Genomic DNA was extracted following the CTAB method. The internal transcribed spacer (ITS) region of rDNA was amplified using ITS-1 and ITS-4, resulted in a PCR amplicon of approximately 560 bp (Fig. 3). The amplified ITS sequence was subjected to BLASTn analysis against the NCBI GenBank database. The sequence showed 100 per cent similarity with *Allocanariomyces americanus* isolates bearing accession numbers PV081142.1, PP057660.1, OQ672369.1, MW194291.1, and ON564534.1. The obtained sequence was deposited in the NCBI GenBank, Maryland, USA under the accession number PX352476, confirmed the identity of the isolate as *Allocanariomyces* sp.

Phylogenetic analysis

The phylogenetic analysis resolved the isolate CDMA-1 and *Allocanariomyces* spp. into two principal clades. The first clade (bootstrap = 99) grouped CDMA-1 with *Allocanariomyce* ssp., strains OZ199747.1 and OZ199743.1 forming a strongly supported sub-cluster (73) while also associating with *Allocanariomyces americanus* strain MW244626.1, indicating close evolutionary affinity. The second clade (bootstrap = 100) comprised *Allocanariomyces* sp. strains OZ199778.1 and OZ199744.1 representing a distinct sub-cluster. At the base of this clade, MF947859.1 (*Pythium* sp.) appeared as a divergent lineage with low support (47), suggesting intergeneric divergence. The clear separation of clades reflects intra-generic diversity within *Allocanariomyces* and confirms the genetic relatedness of CDMA-1 to specific reference strains (Fig. 4). These results were in accordance with Mehrabi *et al.,* 2020 [7].

Conclusion

Die-back of chilli is a major constraint in chilli cultivation, causing substantial yield losses under field conditions. The present investigation was undertaken to elucidate the etiology of this disease through cultural, morphological, pathogenic and molecular characterization of the associated pathogen. The isolated fungus exhibited distinct colony morphology and microscopic features and its pathogenicity was confirmed through artificial inoculation and fulfilment of Koch's postulates. Further confirmation through ITS-rDNA sequencing and phylogenetic analysis established its identity as *Allocanariomyces* sp. Therefore, the results of the present study conclusively demonstrate that *Allocanariomyces* sp. is the causal agent of die-back disease in chilli marking the first detailed report of its association with this disease in the surveyed region.

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Fig 1: Symptomatology of die-back (A- Initial necrosis at shoot tips; B- Typical die-back); C and D front and reverse view of culture of *Allocanariomyces* sp. and E- solitary humicola-like conidia

Fig 2: Pathogenicity assay of die-back caused by *Allocanariomyces* sp. A- Control and B- Inoculated

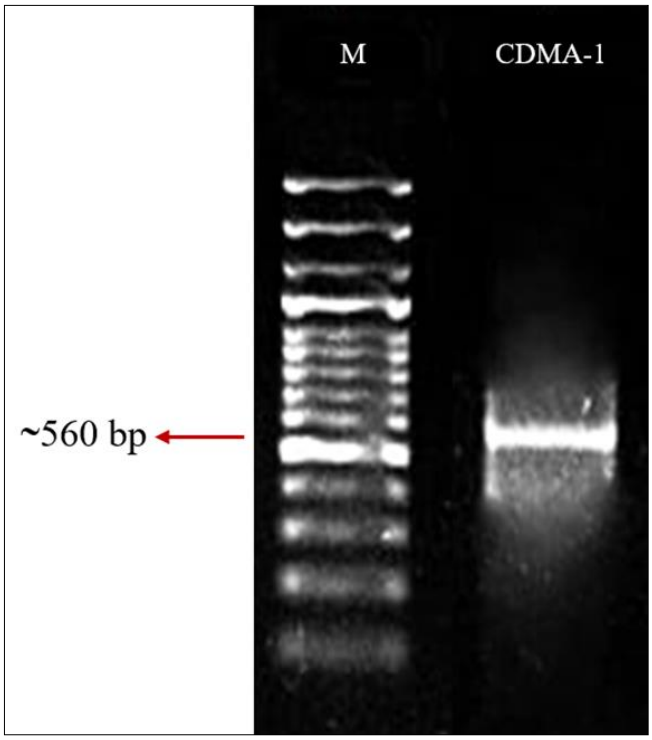

Fig 3: Amplification of ITS region of *Allocanariomyces* sp. causing die-back of chilli

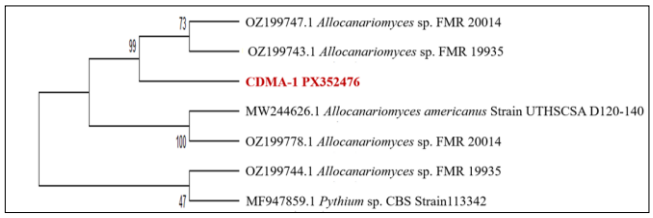

Fig 4: Phylogenetic relationship of *Allocanariomyces* sp. causes die-back of chilli with CBS reference strains based on ITS sequences

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