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MULTIFACETED INVESTIGATION OF *CURVULARIA LUNATA* IN RICE: ISOLATION, IDENTIFICATION AND GRAIN SPOT DISEASE PATHOGENICITY

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ABSTRACT

Grain spot disease in rice, caused by *Curvularia lunata*, poses a significant threat to rice production, leading to substantial yield losses. Aim of the study is to isolate, identify, and evaluate the pathogenicity of *C. lunata* from infected rice grains in Tamil Nadu, India. The pathogen was isolated using single hyphal tip and spore isolation techniques, followed by morphological and molecular identification. Colonies exhibited rapid growth on Potato Dextrose Agar (PDA), with distinct greyish-brown pigmentation. Light and scanning electron microscopy confirmed the characteristic conidial morphology of *C. lunata*. Molecular identification using ITS region sequencing confirmed the species identity, with the isolate showing 89% genetic homology to previously reported *C. lunata* strains. Pathogenicity tests confirmed the fungus as the causal organism of grain spot disease, as inoculated plants developed characteristic lesions, fulfilling Koch's postulates. Growth analysis on different media revealed PDA as the most suitable for optimal mycelial development and sporulation. The findings emphasize the necessity of early detection and effective management strategies to mitigate *C. lunata*-induced grain spot disease in rice. Future research should focus on the genetic diversity of *C. lunata* isolates, host-pathogen interactions, and resistance breeding in rice. Additionally, exploring eco-friendly biocontrol agents and integrated disease management approaches could offer sustainable solutions. This research contributes to the understanding of *C. lunata* as an important rice pathogen and underscores the need for continued surveillance to prevent potential outbreaks in rice-growing regions worldwide.

Key words : *Curvularia lunata*, Grain spot disease, Rice, Pathogenicity, Molecular identification, Disease management.

Introduction

Background of grain spot disease in rice

Curvularia Boedijn belongs to Pleosporaceae, Pleosporales, Pleosporomycetidae, Dothideomycetes, Pezizomycotina, Ascomycota (Boedijn 1933; Sivanesan, 1987; Hyde *et al*., 2013). Species of *Cochliobolus* described by Drechsler (1934), which possess an asexual stage in *Curvularia* as identified by Boedijn (1933), are significant plant pathogens, particularly grasses (Poaceae). These fungi are associated with diseases in over 60 host genera (Sivanesan, 1987; Manamgoda *et al*., 2011, 2012; Tan *et al*., 2014). *Curvularia lunata* was first described as *Acrothecium lunatum* from decaying sugarcane leaves in Java (Wakker, 1898). Boedijin (1933) transferred this species to his newly erected genus *Curvularia* and chose it as the type species, citing new isolations from the air, mango fruit,and other substrates (Manamgoda *et al*., 2012).

Objective of the study

Isolating and identifying pathogens from rice plants involves a multi-step approach integrating morphological and molecular techniques, followed by pathogenicity assays to confirm virulence. Initial isolation includes surface sterilization of symptomatic tissues and culturing on selective media to observe colony morphology, growth rates and pigment production. Morphological


Image 1 : Grain spot symptoms on *Oryza sativa*.

characterization is supplemented with microscopy to assess structural differences. Molecular approaches, such as PCR amplification and sequencing of conserved regions, are critical for precise identification, resolving taxonomic ambiguities. These integrated approaches ensure accurate identification and inform management strategies, such as breeding resistant cultivars or developing biocontrol solutions.

Overview of *Curvularia lunata* and its role as a plant pathogen

The research conducted by AbdElfatah *et al*. (2021) showed that *Curvularia lunata*, a previously under-recognized pathogen in the context of tomato early blight (EB) in Upper Egypt. While *Alternaria* spp., particularly *Alternaria solani* and *Alternaria alternata*, have long been known to cause EB in tomatoes, the role of *C. lunata* has been largely overlooked. Though, *C. lunata* has been associated with tomato fruit rot and leaf spots in other regions, such as India and Pakistan, its involvement in early blight of tomato disease found in Egypt had not been documented. Through molecular characterization of pathogenic fungi using PCR with ITS primers and consequent sequencing of PCR amplicons, we identified *C. lunata* as a key pathogenic species responsible for EB symptoms observed in infected tomato plants from Assiut and Sohag. *C. lunata* causing early blight of tomato in Egypt is marked as first report marking an important addition to the understanding of the pathogens responsible for this widespread disease. *C. lunata* exhibited significant pathogenicity, with lesions appearing on the lower surface of leaves and scattering as the plants matured, consistent with typical early blight symptoms observed in the literature. This study not only highlights the pathogenic potential of *C. lunata* but also emphasizes the need for further investigation into the distribution and management of this pathogen in tomato crops in Egypt and beyond.

Previous studies on *Curvularia lunata* in rice and other crops

Curvularia lunata is one of the most destructive ubiquitous pathogens responsible for stem blight, leaf spot, leaf blight, root rot and necrotic rot in rice, spinach, strawberry, and switchgrass (Bisht *et al*., 2016; Gupta *et al*., 2017; Liu *et al*., 2019; Khan and Javaid, 2020).

Materials and Methods

Infected Sample collection: Description of rice fields, rice varieties and sampling methods

Disease specimens were collected from rice plants from July 2023 to April 2024 in Cuddalore districts of Tamil Nadu. Grains that exhibited symptoms samples were collected randomly per plant and minimum of 10 symptomatic plants were analysed per area. Percent disease incidence (PDI) was calculated by the observation symptoms collected and analyse the percentage of disease incidence. A minimum of thirty observation were made per plant. Use a standardized scoring system to assess disease severity and prevalence across different fields IRRI (2013) Standard evaluation system for rice (SES). Record GPS coordinates using a smartphone. The disease specimen was collected in a self-sealing bag to the laboratory for pathogen isolation. (Nganga *et al*., 2022).

Pathogen Isolation: Techniques used for isolating *Curvularia lunata* from infected rice

The pathogens were isolated from rice plants using the single hyphal tip technique (Gupta *et al*., 2020). From infected leaf tissue small bits (2 to 3 mm pieces) were detached from the border of leaf abrasions, surface sanitized with 1% sodium hypochlorite solution for 1 minutes or ethanol (70%) for 30 sec, washed with uncontaminated distilled water for three times by using sterilized paper and air dried it. The sections (five sections per plate) were then cultured onto a potato dextrose agar medium (PDA**;** MO96, dissolved 39g in 1000 ml distilled water). PDA contains 200g of potatoes, 20g of dextrose, and 15g of agar in 1,000ml of water, final pH of 5.6 ± 0.2. The cultures were incubated in the Biological Oxygen Demand (BOD) at 25^{o} C in the dark condition. The growing edges of the mycelium were observed and transferred into PDA-amended plates under aseptical condition.

Furthermore, the pathogens were isolated from rice plants by using the Manamgoda *et al*. (2012) single hyphal spore isolation method. In wet chamber (Environmental Test Chamber, Corporation, Japan) disease specimen were incubated for a period of 48 h. Spores have been extracted from the sporulating samples and observed under a stereo microscope. 400 micro liter of sterilized distilled water were mixed to the spores. Through a sterile pipette the mixture spores and sterilized distilled water has been shifted to water agar (WA) plates. Spores were permitted to germinate on the water agar plates for 12 h,

afterwards which they were individually moved to a fresh PDA media. Each isolate was preserved at – 80 degrees Celsius in an ultra-low temperature freezer (Corporation, Japan) containing 15% glycerol.

Morphological Characterization: Methods for identifying the pathogen based on cultural and morphological characteristics (colony growth, colour, conidia shape, etc.)

Mycelial disc (5mm diameter) was gently moved from active cultures to autoclaved PDA amened plates and incubated at 25°C in the absence of light. The growth rate, the colony appearance, and texture were examined as well as the pigmentation on both the top and reverse plates. To conduct morphological analysis, a pinch of mycelium was placed on glass slide with sterile distilled water. An iScope trinocular fluorescence microscope (Euromex, Netherlands IS 3153 PLFi/3) equipped with a Dc 20000i-microscope camera and differential interference contrast was used to measure morphological charateristics such as conidial length, width, septation, conidiophore length, width and conidia attachment to condiophores (Euromex Image-Focus-Alpha analysis software (Euromex, Netherlands) measured more than 30 conidia of an isolate.

Microscopic Observation: Scanning Electron Microscopy (SEM) for detailed structural analysis of the pathogen

Morphological characteristics were also observed in a scanning electron microscope (SEM) with EDS (JEOL-JSM-IT 200) in the Centralised Instrumentation and Service Laboratory, Annamalai University. The mycelium samples were processed according to Khan and Javaid (2020). Before being separated into three 1cm pieces, the mycelium was placed in SEM stubs. The mycelium was treated with glutaric dialdehyde (4%), followed by three washes in 0.1M cacodylic sodium trihydrate buffer. It was then fixed for one hour in a 1% osmium tetroxide solution, treated with sodium trihydrate buffer, and subsequently dehydrated in pure ethanol before being passed through acetone. Finally, the mycelium was mounted on a stub for SEM imaging.

Molecular Identification: DNA extraction, PCR, sequencing and genetic analysis for species confirmation

DNA extraction

Manamgoda *et al*. (2012) revealed that the cetyltrimethyl ammonium bromide (CTAB) method for genomic DNA extraction. A 100 mg sample of fresh mycelium was scraped from the surface of the medium

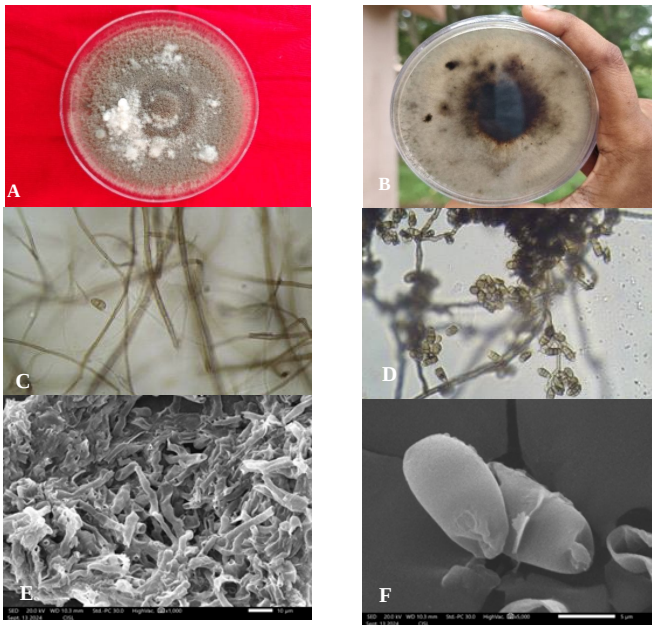

Image 2 : Cultural and morphological characterization of *Curvularia lunata* isolated from *Oryza sativa*. Colony morphology in front (A) and in reverse (B) on PDA (C, D) light microscopy image of hyphae and conidiospore (E, F) Scanning electron microscopy image of hyphae and conidiospore.

and placed in 1.5 ml microcentrifuge tubes. To this, 600 µl of preheated 2× CTAB extraction buffer [2% (w/v) CTAB, 100 mM Tris-HCl, 1.4 M NaCl, 20 mM EDTA, pH 8.0] and 0.2 g of sterilized quartz sand were added. The mycelium was then ground for 3 to 5 minutes using a sterilized glass pestle. The mixture was gently agitated and incubated at 60°C with periodic swirling. Following a rapid centrifugation for 15 minutes at 12,000 rpm, the solution underwent constant chloroform-isoamyl extraction. After DNA precipitation using isopropanol, the solution was centrifuged again for 15 minutes at 12,000 rpm. The residue was treated with 70% ethanol and centrifuged for 5 minutes at 12,000 rpm. After 20 minutes, the DNA was re-suspended in 70 µl of Tris-EDTA (TE) buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and stored at –20°C. The DNA concentration and quality were assessed using a NanoDrop™ 1000 Spectrophotometer (Thermo Fisher Scientific)

PCR amplification

The internal transcribed spacer (ITS) of rDNA was used to amplify each isolate using universal primers ITS 1 (5'-TCCGTAGGTGGACCTGCGG-3') as forward primer and ITS 4 (5'-TCCTCCGCTTATTGATATGC-3') as a reverse primer (White *et al.*, 1990) were used to amplify the 5.8S and to flank ITS regions. An Eppendorf Master cycler Nexus Thermal Cycler (Germany) was used to amplify the DNA fragments. 2.5 µl of 10× Taq

buffer, 0.5 µl of 25 mM $MgCl_2$, 2.0 µl of ITS-1 forward primer, 2.0 µl of ITS-4 reverse primer, 0.5 µl of 100 mM dNTPs, 0.125 µl of Taq DNA polymerase, 14.375 µl of sterile distilled water and 3 µl (100 ng) of DNA sample were all comprised in the 25 µl of reaction volume of distilled water. In every amplification, negative control was introduced, in which the template DNA was switched out for an equivalent volume of distilled water. The following criteria were used for performing PCR with the starting with denaturing at 94°C for 4 minutes, for 2 minutes at 94°C 40 cycles of denaturation occurred and annealing starts with 52°C for 2 min and extension at 72°C for 2 min finally the extension at 72°C for 7 minutes.

Gel electrophoresis

A 1.5% agarose gel 0.5× TAE buffer (20 mM Tris-acetate pH 8.0, 0.5 mM EDTA) used to electrophorese the PCR products. UV illumination light and ethidium bromide (10 mg/ml) staining were used to visualized DNA in a gel documentation system. By correlating the PCR products size to a 100 base pair DNA ladder, a known DNA marker, its size was determined. A gel documentation system captured the ITS primmer sequenced PCR products subsequently, a slightly of alkaline buffer (5 mM Tris-Cl, pH 8.5) was used to elute the pure DNA under low strength condition.

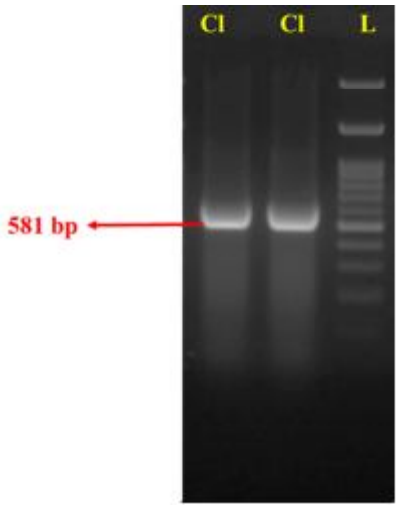

Image 3 : Agarose (1.5%) gel electrophoresis of PCR using ITS1 and ITS4 primers fungal sample.

DNA sequencing and alignment

The amplified product was sequenced by a commercial sequencing provider (Medauxin, Bengaluru, India). The DNA sequence obtained was initially analyzed using the DNASTAR SeqMan Ultra (DNASTAR Incorporation, Madison, USA). The DNA forward and reverse sequences were then aligned to generate consensus sequences. MEGA 11 software was used to perform the alignment for each gene (Tamura *et al.*, 2021). The isolated ITS sequence was submitted to the NCBI database (http://www.ncbi.nlm.nih.gov), where it was assigned an accession number. To compare ITS nucleotide rDNA sequences from the pathogen, isolate, BLASTn (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to search the NCBI database. ITS sequence was compared to other isolates infecting different crops extracted from GenBank.

Phylogenetic analysis

The phylogenetic tree was created with MEGA 11 software using the maximum likelihood (ML) approach with 1000 bootstrap replications (Tamura *et al*., 2021).

Pathogenicity Testing: Inoculation methods, symptoms observation and pathogenicity assessment

The pathogenicity assay was conducted according to the method described by Verma and Gupta (2010). Rice plant leaves have been decontaminated with sodium hypochlorite (1%) for 1 minute 30 seconds. Then they were washed three times with sterilized water. Rice plants grown in earthen pots were inoculated with 6 ml of isolate conidial suspension by spraying (1.0×10^6 conidial/ml). The control plants were sprayed with the same amount of sterilized water. The plants were enclosed with plastic bags to retain high humidity for 48 hours, then transported into a glasshouse under natural sunshine conditions at 28±3° C. Isolate was tested with five replicates in each experiment. The experiment was conducted twice. Disease development was monitored on a daily basis. Re-isolation of pathogen from inoculated plant lesions, and its structural features were compared with the original isolate. The pathogenicity assay was also performed by inoculating test isolate mycelial disc (3 mm) onto healthy plant leaves, then left in a humid chamber at 25°C for 48 hours. Control plant leaves have endured in the same conditions. The experiment was carried out twice. Disease development was recorded daily. The pathogen was re-isolated from inoculated which produced leaf lesions and morphological characteristics compared with the original isolate. This method was proposed by Rocha *et al.* (2004).

Medium Growth Analysis: Testing different growth media to assess pathogen growth

Five-millimetre discs of isolates taken from axenic cultures were positioned at the center of petri plates containing media of four different composition : Potato Dextrose Agar (PDA; MO96, suspend 39g in 1000 ml distilled water) Czapek Dox Agar (CDA; M075, suspend 49.01 g in 1000 ml distilled water), Oat Meal Agar (OMA;

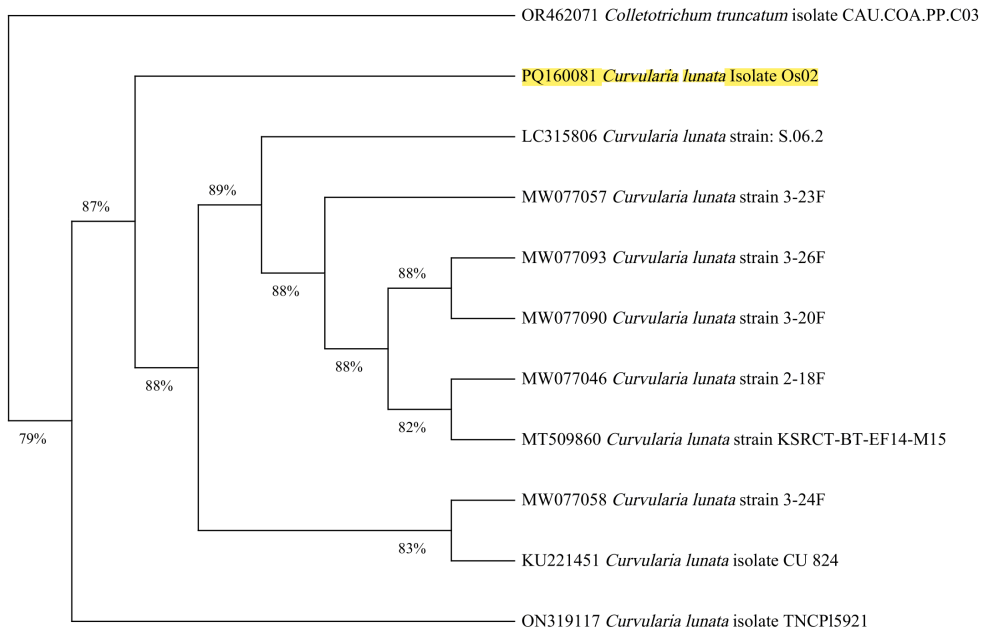

Fig. 1 : Maximum likelihood (ML) tree generated from analysis of ITS in the *Curvularia lunata* on *Oryza sativa*. ML bootstrap values > 90% are displayed above or below each isolate. The isolate collected and identified in this study is in yellow colour.

Image 4 : Pathogenicity test by *Curvularia lunata* conidial suspension sprayed on rice leaf. (A) Uninoculated leaf (B) Typical symptoms appear on inoculated leaf.

M397, suspend 72.5 g in 1000 ml distilled water) and Corn Meal Agar (CMA; M146, suspend 17 g in 1000 ml distilled water). The plates were then incubated at 25°C in the dark with colony growth was monitored regularly. CDA consists sucrose 30 g, sodium nitrate 2 g, dipotassium phosphate 1 g, magnesium sulfate 0.5 g, potassium chloride 0.5 g, ferrous sulphate 0.01 g, 15 g agar in 1,000 ml of water with final pH of 7.3 ± 0.2. OMA procedure: oatmeal 60 g, 12.5 g of agar in 1,000 ml of water, and a final pH of 7.2 ± 0.2. CMA contains cornmeal 50 g, 15 g agar in 1,000ml of water with final pH of 6.0 ± 0.2.

Results

Isolation and identification of *Curvularia lunata*: Morphological and molecular identification results

A fungal isolate was obtained from infected rice grains and colony was grown on PDA, which reached 82 mm

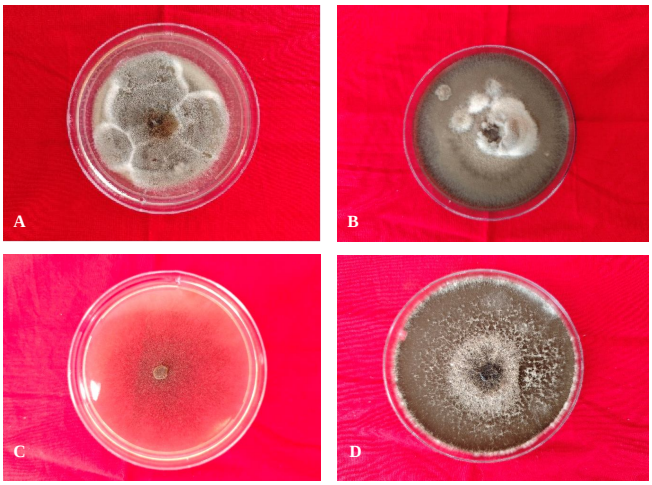

Image 5 : Colony morphology of *Curvularia lunata* on different media. (A) Potato Dextrose Agar, (B) Czapek Dox Agar, (C) Corn Meal Agar and (D) Oat Meal Agar media.

at 25°C in seven days. Colonies were fast-growing, greyish with white tinch, reverse mouse grey at the center, and brown at the margin. Hyphae septate, branched, subhyaline to brown, smooth to asperulate, up to 5 µm wide, anastomosing. Conidiophores were reddish-brown, septate, unbranched and 4.5-9 µm thick. Conidia were fusiform, cylindrical, 3-septate, basal and apical pale brown, central cells bring larger and darker, 5.4 -14.6 × 12.22 - 22.6 µm. Light microscope and SEM images were revealed that conidiophores bearing conidia separately and in chains. PCR was used to amplify the rDNA region, and the 581 bp sequence of Os02 (*Oryza sativa*-02)

was submitted to GenBank under Accession No. PQ160081. The isolate exhibited 89% genetic homology with previously reported C. *lunata* to isolate (MW077058).

Pathogenicity Assessment: Results from pathogenicity testing on rice

Spraying isolated fungal conidial suspension onto plants. The control plants were treated with sterile water. The inoculated and control plants were sealed with a plastic bag for 48 hours. Control plants remained symptoms-free, but infected leaves developed purple dark lesions on leaf sheath after seven days for completing Koch's postulates. The infected plants were reisolated, and it appeared to be the same fungus.

Growth Characteristics on different Media: Comparison of pathogen growth on various media

The colony morphology of *Curvularia lunata* was examined on different culture media, revealing distinct variations in hyphal growth, colony texture and pigmentation. Among the tested media, Potato Dextrose Agar (PDA) exhibited the most prolific mycelial growth, characterized by a fast-spreading, cottony and fluffy colony with a grayish-black pigmentation and a white peripheral margin. Sporulation was abundant, indicating the suitability of PDA for fungal propagation. On Czapek Dox Agar (CDA), the fungal colony displayed a compact, dark, and velvety texture with a well-defined center. The growth was slower compared to PDA, but sporulation was moderate. The colony surface appeared slightly raised with a dark pigmented core. Corn Meal Agar (CMA) supported moderate fungal growth, forming a dull brownish-black colony with a smoother texture. The mycelial mat was dense but lacked aerial hyphae, leading to a relatively flat appearance. Sporulation was relatively lower compared to PDA and CDA. Oat Meal Agar (OMA) supported uniform radial growth with a dark, woolly and dense mycelial mat. The colony exhibited a deep grayish-black color, and sporulation was moderate to high. The texture was more compact than that observed on PDA, and the pigmentation was consistently darker. Overall, *Curvularia lunata* exhibited optimal mycelial growth on PDA and CDA, with significant sporulation observed in PDA. The colony morphology, including pigmentation and texture, varied based on the nutrient composition of the medium. These findings align with previous studies highlighting PDA and nitrogen-rich media as ideal for fungal growth and sporulation.

Discussion

Interpretation of morphological, molecular, and SEM findings

The present study demonstrated that *Curvularia lunata* exhibits distinct growth patterns on different culture media. Among the tested media, Potato Dextrose Agar (PDA) supported the highest mycelial growth, forming a cottony, fast-spreading colony with abundant sporulation, which aligns with previous findings (Lal *et al*., 2014). Czapek Dox Agar (CDA) produced compact, dark colonies with moderate growth and sporulation, showing slightly slower development than PDA but still supporting fungal propagation. Corn Meal Agar (CMA) resulted in moderate fungal growth with a dense but smoother colony texture, lacking aerial hyphae and exhibiting lower sporulation compared to PDA and CDA. Oat Meal Agar (OMA) facilitated uniform radial growth with a woolly texture and deep grayish-black pigmentation, with sporulation levels between those observed on PDA and CMA. Overall, PDA provided the most favorable conditions for *C. lunata* growth and sporulation, followed by CDA, while CMA and OMA supported moderate development. These findings emphasize the role of media composition in fungal propagation, supporting previous research on the optimal conditions for *C. lunata* cultivation (Lal *et al*., 2014 and Rao *et al*., 2020).

Discussion of pathogenicity results and their impact on rice health

The pathogenicity of *Curvularia lunata* was confirmed through various inoculation methods across different host plants. The current study validated the pathogenic nature of *C. lunata* on rice leaves under controlled conditions, supporting previous findings on its ability to cause leaf spot diseases in cotton (Joshi *et al*., 2023), *Aloe vera* (Avasthi *et al*., 2015) and maize (Chang *et al*., 2020). The inoculated plants produce symptoms, as well as the pathogen's successful re-isolation indicate its role in disease manifestation and support Koch's postulates. The results are consistent with Avasthi *et al*. (2015), who demonstrated *C. lunata* pathogenicity on *A. vera* leaves, where round, water-soaked lesions developed within four days, later progressing to necrotic dark brown lesions. Similar findings were reported in maize by Chang *et al*. (2020), where isolates HNWB-131 and HNWB-185 exhibited significant disease severity, particularly in Huangzao 4. In cotton, Joshi *et al*. (2023) observed symptoms on artificially injured plants, confirming the role of *C. lunata* in causing Curvularia leaf spot. In the present study, rice plants inoculated with *C. lunata* showed symptoms of purple-dark lesions on

the leaf sheath within seven days, corroborating Rocha *et al*. (2004). The successful re-isolation of the pathogen and morphological consistency with the original isolate further affirmed its pathogenicity. Control plants remained symptom-free, reinforcing the necessity of fungal inoculation for disease onset.

Analysis of the best media for culturing *Curvularia lunata*

The study revealed that *Curvularia lunata* exhibits varying growth and sporulation patterns depending on the culture medium. Among the tested media, Potato Dextrose Agar (PDA) supported the highest mycelial growth and sporulation, consistent with previous findings (Lal *et al*., 2006). Czapek Dox Agar (CDA) produced compact, dark colonies with moderate growth, aligning with earlier studies indicating its suitability for fungal maintenance (Rao *et al*., 2020). Corn Meal Agar (CMA) showed moderate growth with a dense but smooth colony texture, while Oat Meal Agar (OMA) facilitated uniform radial expansion with deep pigmentation, supporting observations by Dhingra (1982). Overall, PDA and CDA proved to be the most effective for *C. lunata* growth and sporulation. These findings reinforce the importance of selecting nutrient-rich media for optimal fungal propagation.

Conclusion

Grain spot disease in rice, caused by *Curvularia lunata*, is a significant concern in rice cultivation due to its detrimental effects on grain quality and yield. This study highlights the pathogenic nature of *C. lunata*, its symptoms, and its impact on rice production. The characteristic dark brown to black spots on rice grains not only reduce market value but also concession seed viability, leading to economic losses for farmers. The study demonstrates that environmental factors such as humidity and temperature influence the spread and severity of *C. lunata* infections. Understanding these factors can help you to implement effective management control. Traditional disease management methods, including cultural practices like crop rotation and sanitation, along with biological and chemical control measures, are essential in mitigating the spread of grain spot disease. Future research should concentrate on breeding of resistant rice varieties and integrated disease management approaches that include genetic resistance, biocontrol agents and environmentally friendly fungicides. Additionally, further investigation of molecular mechanisms underlying *C. lunata* pathogenicity will provide deeper insights into effective disease control. By implementing comprehensive management strategies and fostering continued research, the impact of grain spot disease in rice can be minimized, ensuring better yield stability and food security for rice-dependent populations worldwide.

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