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RESEARCH ARTICLE

Isolation and multidimensional characterization of *Colletotrichum gloeosporioides* (Penz. & Sacc.) associated with anthracnose in groundnut (*Arachis hypogaea* L.)

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Abstract

Groundnut (*Arachis hypogaea* L.) is most popularly called as the 'King of oil seeds' and it is a highly valued leguminous crop in India and worldwide. Groundnut productivity is declining due to multiple biotic and abiotic stresses that hinder effective cultivation and cause substantial yield losses. Anthracnose, a destructive fungal disease incited by *Colletotrichum* species, has become a significant threat to groundnut cultivation in key growing regions of Tamil Nadu. The disease manifests initially as small, water soaked and yellowish spots along the leaf margins, which progressively expand and develop into well defined, circular brown lesions with a distinct yellow halo. As the infection advances, extensive foliar damage occurs, ultimately leading to substantial yield losses. The disease, exacerbated by warm, dry conditions and airborne spores during the rainy season. This study investigated the morphological and growth characteristics of ten *Colletotrichum gloeosporioides* isolates collected from different locations. The isolates exhibited distinct variations in mycelial growth, colony pigmentation, zonation patterns and sporulation capacity. Notably, isolate Cg1 exhibited the highest mycelial growth. Morphologically, the isolates displayed diverse features, including a fluffy white appearance, cottony mycelium and variations in sporulation. Additionally, differences in conidial size and shape were noted. The pathogen exhibited optimal growth under a 12 hrs light and 12 hrs dark cycle, at a temperature range of 25-30 °C and a pH of 5.0. This study emphasizes the pivotal influence of morphological and physiological parameters influencing the development and growth of *Colletotrichum gloeosporioides* isolates.

Keywords: anthracnose; *Colletotrichum gloeosporioides*; groundnut

Introduction

Groundnut (*Arachis hypogea* L.), colloquially known as peanuts, is an indispensable and versatile crop that has firmly embedded itself in the agricultural practices of nations worldwide. The groundnut is a member of the legume family, *Fabaceae* and is renowned with agricultural richness and economic significance for its dual-purpose nature as both oil seed and a protein rich pulse. India ranks as the world's second largest producer of groundnut, with an annual yield exceeding 10 million metric tons and an average productivity of 1863 kg per hectare (1). Gujarat dominates production, accounting for 33 %, succeeded by Rajasthan (21 %), Tamil Nadu (14 %), Andhra Pradesh (7 %) and finally Telangana (5 %). India constitutes 36 % of global groundnut exports, with major destinations being Indonesia, Vietnam, Malaysia, UAE, Philippines and Thailand (2). Groundnut cultivation, from planting to storage, grapples with several disease constraints that pose significant challenges to yield and crop health. More than 55 pathogens reported on affecting groundnut including bacterial, fungal, viral, nematode and phytoplasmas.

Anthracnose, a formidable fungal disease caused by *Colletotrichum* species, stands as a considerable emerging threat to groundnut farming worldwide (3). This insidious pathogen, primarily *Colletotrichum* spp., poses a substantial menace to groundnut farming, severely affecting both quality and yield. The disease manifests as distinctive lesions on leaves, stems and pods, presenting a complex challenge for the agricultural sector (4). This disease affects various parts of the plant, which include young leaves, stem portion, inflorescences and pods. The key identifying symptoms are the presence of numerous oval or irregular brown to dark brown circular or angular spots of varying sizes scattered across the leaf surface.

When there is moisture, these spots rapidly expand and develop into brown necrotic region that measures about 2.0-2.5 mm in diameter. As the spots enlarges, they become ruptured and eventually fall off (5). Due to varying crop stages at the time of infection and disease severity, yield loss, ranging from 4-35 % have been documented in various crops affected by anthracnose disease (6). Despite the economic significance of this disease, research efforts addressing groundnut anthracnose have been notably scarce. Recognizing its severity and the limited past research on its control, it became imperative to undertake investigations on the pathogen. Thus, the present study aimed to address this gap and contribute to understanding and management of groundnut anthracnose effectively. Thus, the objectives designed for the present study are:

i) To conduct a systematic survey to isolate *Colletotrichum gloeosporioides* from groundnut-growing regions across Cuddalore district, Tamil Nadu.

ii) To characterize the *C. gloeosporioides isolates* using morpho-cultural, physiological and molecular approache.

Materials and Methods

Sampling of infected plant tissues

A roving survey was conducted during 2023-2024 to collect groundnut leaf samples exhibiting typical leaf spot symptoms from various villages in the Cuddalore district of Tamil Nadu. In each village, four fields were selected and within each field, four randomly marked plots, each covering an approximate area of ten square meters, were surveyed and the percent disease index was determined using the following formula (7).

Percent disease index =

$$\frac{Sum\ of\ the\ individual\ disease\ ratings}{Number\ of\ Pods/leaves\ observed} \times \frac{100}{Maximum\ disease\ grade}$$

The disease severity was assessed based on a grading scale corresponding to the percentage of leaf area infected. A score of 0 indicated no infection, while a score of 1 represented 1-10 % leaf area infection. Leaves with 11-25 % infection were assigned a score of 2, whereas those with 26-50 % infection were graded as 3. A more intense infection level, ranging from 51-70 %, was assigned a score of 4, while the most severe infection, affecting 71-100 % of the leaf area, was categorized as grade 5.

Isolation of pathogen

The fungus was isolated using the traditional tissue isolation method. Five days old diseased groundnut leaves were surface sterilized (8). Leaf segments (5 mm), including the infection margins, were sterilized in 70 % ethanol for 1 min, rinsed thrice with distilled water and placed on Potato Dextrose Agar (PDA) medium supplemented with streptomycin sulfate to inhibit bacterial contamination. The plates were incubated at 28 ± 3 °C for eight days, after which pure cultures were subcultured and preserved at 4 °C for further analysis (9-11).

Morpho-cultural characteristics of the isolates

Approximately 15 mL of sterile PDA was dispensed into aseptic Petri dishes, which were then left to solidify. 9 mm mycelial disc of *Colletotrichum* culture was carefully placed at the centre of each plate and incubated at a standardized temperature of 28 ± 3 °C with a relative humidity (RH) of 60-70 % over a duration of seven days. Subsequent to incubation, the radial expansion of the fungal colony was systematically assessed by measuring its growth in two perpendicular directions to ensure accuracy and consistency in observations. In addition, the mycelial colour, morphological variation of mycelia, conidia, sporulating structures, size and population of conidia were measured and counted (9, 12).

Pathogenicity

Earthen pots, each measuring 30 cm in diameter, were filled with 5 kg of a sterilized potting mixture consisting of sand, red soil and FYM in a uniform 1:1:1 ratio. Groundnut seeds of the VRI-2 variety underwent surface sterilization by immersing them in 0.1 % of sodium hypochlorite solution for 2 min. Subsequently, the samples were thoroughly rinsed with sterile distilled water to remove any remaining traces of disinfectant. The treated seeds were sown at a density of 2–3 seeds/pot. All experimental pots were maintained under greenhouse conditions to ensure optimal plant growth. A conidial suspension of *Colletotrichum* spp. was prepared from a 10 day old actively growing fungal culture using sterile distilled water. The spore concentration was meticulously adjusted to 2.5×10^6 conidia per mL with the aid of a hemocytometer to ensure uniform inoculum density. 20 mL of the prepared suspension were evenly sprayed onto 25 day old groundnut plants to facilitate infection. Post-inoculation, the plants were relocated to a humid chamber maintained at 100 % RH and a controlled temperature range of 26–28 °C for 15 days to promote disease development. As a negative control, a separate set of groundnut plants was sprayed with sterile distilled water. The onset and progression of disease symptoms were systematically assessed 15 days after inoculation (9, 13).

Morphological characterization using scanning electron microscopy (SEM)

To examine the structural characteristics of the fungal hyphae, SEM imaging was performed. For sample preparation, an actively growing fungal culture was subjected to fixation overnight at 28 °C in a 0.05 M phosphate buffer solution containing 4 % glutaraldehyde to preserve cellular structures. The following day, the fungal mat underwent three consecutive washes with phosphate buffer to remove any residual fixative. Subsequently, a graded ethanol dehydration process was carried out, with each stage lasting 15 min to guarantee thorough dehydration and elimination of moisture. Following dehydration, the samples were subjected to critical point drying using CO_2 for 5 min to prevent structural collapse. The dried fungal specimens were then carefully mounted onto aluminum stubs to facilitate imaging. To optimize conductivity and enhance image clarity, the samples were coated with a thin carbon layer using a Polaron E-500 sputter coater. The prepared specimens were immediately examined under a SEM at an accelerating voltage of 15 kV to capture high-resolution micrographs of fungal hyphae. The SEM analysis was conducted at the CSIL, Department of Physics at Annamalai University.

Molecular characterization of *C. gloeosporioides*

DNA extraction

Fungal strains were cultivated in 100 mL of potato dextrose broth and incubated at 28 ± 2 °C for seven days to facilitate robust mycelial growth. Once the incubation phase was finalized, the mycelial biomass was meticulously collected and processed for genomic DNA extraction utilizing a modified CTAB buffer protocol, ensuring the isolation of high integrity DNA, meticulously extracted for advanced molecular studies. To begin the extraction process, approximately 2 g of mycelial tissue was frozen in liquid nitrogen and finely pulverized using the sterile pestle and mortar. A measured 200 mg of the ground mycelium was transferred into a 2.0 mL Eppendorf tube and supplemented with 1 mL of an extraction buffer composed of 20 mM EDTA (pH 8.0), 2 % cetyltrimethylammonium bromide (CTAB) and 0.1 % β-mercaptoethanol to facilitate cell lysis and protein denaturation (14). The homogenized mixture was vigorously agitated and incubated at 65 °C for 10 min to enhance nucleic acid release. Subsequently, a chloroform:isoamyl-alcohol (24:1) solution was introduced to the lysate, promoting phase-separation. The sample was subjected to centrifugation at 10000 rpm for a duration of 10 min and the supernatant was meticulously extracted to retain the aqueous phase. DNA precipitation was induced by the addition of equal volumes of 5 M NaCl and ice cold Isopropanol was then added and the mixture was incubated at 65 °C for immediate precipitation or stored overnight at –20 °C to maximize DNA recovery. The pelleted DNA was obtained by centrifugation at 13000 rpm for 10 min and subsequently washed twice with 70 % ethanol to eliminate any remaining impurities. Ultimately, the purified DNA was dissolved in 50 µL of TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) to preserve its stability and integrity for downstream applications and stored under appropriate conditions for subsequent molecular analyses.

PCR amplification and sequencing of 18S rDNA of *C. gloeosporioides*

The extracted genomic DNA was precisely quantified using a NanoDrop spectrophotometer to assess its purity and concentration. Subsequently, its integrity and quality were evaluated through electrophoretic separation on a 0.8 % agarose -gel, ensuring suitability for downstream molecular applications. A well-defined high molecular weight DNA band was detected, validating the integrity of the extracted DNA. This purified DNA was then utilized for the PCR amplification of the ITS region, ensuring precise molecular characterization. The amplification process was performed using the universal primers ITS1-F and ITS4-R, generating a distinct amplicon measuring approximately 650–700 base pairs in length. The PCR-amplified fragments were examined through agarose gel electrophoresis to confirm their presence and integrity, under UV illumination to confirm successful amplification. For precise sequencing, both forward and reverse reactions were carried out using the corresponding primers in conjunction with the BigDye Terminator v3.1 cycle sequencing kit. The sequencing process was executed on an ABI-3730xl genetic analyzer to ensure high-accuracy nucleotide identification. The obtained sequences were subjected to BLAST analysis and the top-matching sequences with the highest identity scores were selected. Multiple sequence alignment was carried out using BioEdit v7.2 and ClustalW to assess genetic similarity and divergence among the isolates. A distance matrix was computed and a phylogenetic tree was inferred using the neighbor-joining method in MEGA X 11, facilitating the assessment of evolutionary relationships among the analyzed sequences. The final consensus sequences were submitted to NCBI GenBank for accession number assignment, enabling their inclusion in public genetic databases for further comparative studies.

Physiological studies on the mycelial proliferation of *C. gloeosporioides* (Cg1)

Influence of various solid culture media

Trials were performed to assess the differential growth response of *C. gloeosporioides* (Cg1) on various culture media, namely PDA, Czapek's Dox Agar, Corn Meal Agar, Oat Meal Agar, Potato Sucrose Agar, Beetroot Agar and Carrot Agar. Each culture medium was prepared following standard protocols, sterilized in an autoclave and supplemented with antibiotics prior to pouring into sterile 9 cm Petri plates to prevent bacterial contamination. Once solidified, a 5mm diameter culture disc of *C. gloeosporioides* (isolate Cg1) was excised from a 10 day old culture utilizing a sanitized cork borer and centrally placed on the agar surface. The inoculated plates were incubated under ambient laboratory conditions. Radial colony growth was monitored by drawing 2 perpendicular reference markings on the underside of the Petri dishes, intersecting at the centre. Measurements were recorded at 24 hr intervals until complete coverage of the medium was achieved in any treatment. Sporulation data were assessed after 10 days of incubation using a graded meter chart. For this purpose, conidia were harvested from the culture plates by adding sterile water and spore density was assessed using a hemocytometer (15). The experiment followed a completely randomized design with three replications, ensuring statistical validity of the results (16).

Influence of various liquid culture media

Seven distinct liquid media, Potato Dextrose Broth, Czapek's Dox Broth, Corn Meal Broth, Oat Meal Broth, Potato Sucrose Broth, Beetroot Broth and Carrot Broth were meticulously prepared and analyzed to assess their influence on the mycelial biomass accumulation of *C. gloeosporioides* (Cg1). For this study, 10 mL of each sterilized broth medium was aliquoted into 250 mL Erlenmeyer flasks and subjected to autoclaving to ensure sterility. Following cooling, a 5mm mycelial plug of *C. gloeosporioides* (Cg1), excised from a 10 day old culture plate using a sterile cork borer, was aseptically introduced into each flask. The inoculated cultures were incubated under controlled ambient laboratory conditions. After a 15 day incubation period, the fungal mycelium mat, which had developed on the surface of the liquid medium, was carefully retrieved by vacuum filtration through a Buchner funnel lined with pre weighed Whatman No.1 filter paper. To achieve complete desiccation, the harvested biomass was oven dried at 70 °C for 24 hr. The final dry weight of the fungal mass was calculated using the prescribed formula (16):

Dry weight = (weight of filter paper + mycelial weight) - (weight of filter paper)

Influence of Various light regimes

For this experiment, sterilized PDA medium, supplemented with antibiotics to prevent bacterial contamination, was dispensed into 9 cm Petri plates. Once the medium solidified, a

5mm diameter agar disc of *C. gloeosporioides* (Cg1), obtained from a 10 day old culture plate using a sterile cork borer, was carefully and aseptically placed at the centre of each Petri dish. The inoculated plates were subjected to five distinct light exposure conditions: continuous light for 24 hr, complete darkness for 24 hr, alternate light and dark cycles of 12 hr each, 16 hr of light followed by 8 hr of dark and 8 hr of light followed by 16 hr of dark. Each treatment was replicated three times to ensure statistical reliability. Radial colony growth was systematically measured to determine the influence of different light regimes on fungal development (16).

Influence of various temperature and pH

In this investigation, a 5 mm mycelial plug excised from a 10 day old culture of *C. gloeosporioides* (Cg1) was meticulously introduced onto Petri dishes preloaded with autoclaved PDA. The inoculated plates were subsequently subjected to six distinct temperature conditions, 15 °C, 20 °C, 25 °C, 30 °C, 35 °C and 40 °C. To ensure the reliability of the findings, each experimental setup was replicated thrice for statistical validation. Radial colony expansion was measured at 24 hr intervals and conidial germination rates were also recorded to assess the effect of temperature on fungal growth and development. A spore suspension (20–30 spores per low power field) was prepared in sterilized water and a drop was placed on a slide in a humid chamber (moist filter paper in sterilized Petri dishes). Plates were incubated at the specified temperatures and observations were made on mycelial and conidial metrics at 6 hr intervals up to 24 hr (17).

The effect of pH on the optimal proliferation of *C. gloeosporioides* (Cg1) was evaluated using PDA media adjusted to different pH levels 5, 6, 7, 8, 9 and 10. A 5 mm agar disc from a 10 day old fungal culture was aseptically transferred onto Petri plates containing the pH-adjusted media. The culture plates were subsequently maintained at controlled room conditions to evaluate the effect of pH fluctuations on fungal proliferation and morphological development. The treatments were replicated thrice and radial growth was measured and recorded (16).

Grade meter chart

S. No.	Grade	Score	No. of conidia / microscopic field at 100X
1	Excellent	++++	55–70
2	Good	+++	30–55
3	Fair	++	25–30
4	Poor	+	10–25

Statistical analysis

All experimental observations were subjected to statistical analysis using WASP v2.0, a pioneering web-based agricultural statistics software, to quantify variability among treatments. To ensure normality, disease incidence data underwent arcsine transformation before being analyzed through Analysis of Variance (ANOVA). Subsequently, treatment means were differentiated using Duncan's Multiple Range Test (DMRT) within a Completely Randomized Design (CRD), maintaining a 5 % level of significance ($p < 0.05$) for laboratory evaluations (18).

Data visualization

The experimental data were analysed and visualized using Python version 3.7.8, a widely used programming language in scientific computing and data visualization. The NumPy library was utilized for numerical computations and data manipulation, while the Matplotlib library was employed to create detailed and publication ready visualizations, including bar charts and line plots. These graphical representations were customized to include labelled axes, error bars, legends and colour coded elements to enhance clarity and facilitate comparative analysis of the results.

Results and discussion

Survey, isolation and morphological characterization of different *Colletotrichum* spp. isolates

A systematic roving survey carried out during the 2023–2024 rabi season across key groundnut cultivation regions in Cuddalore district, Tamil Nadu, indicated the widespread prevalence of anthracnose, establishing its endemic nature in the area (Table 1). Anthracnose disease manifests across various plant parts, including young leaves, stems, inflorescences and pods. Notably, its identifying symptoms manifest as numerous oval or irregular brown to dark brownish circular, or angular spots of varying sizes dispersed across leaf surfaces. In the presence of moisture, these spots swiftly expand, forming brown necrotic regions measuring approximately 2.0-2.5 mm in diameter (Fig. 1). Among the ten surveyed locations, Sivapuri recorded the highest incidence (37.54 %), followed by Bhuvanagiri (34.73 %), Keerapalayam (30.69 %) and Panruti (27.78 %), with Kammapuram reporting the lowest (5.75 %). The disease incidence was higher in cultivars VRI 2 and VRI 3, particularly during the peg penetration and pod development stages. Native isolates of *Colletotrichum* spp. were obtained from the locations and designated as Cg1 to Cg10. Similar to the present study, a comprehensive and systematic roving survey conducted in the Dindigul district of Tamil Nadu revealed that the severity of anthracnose in legume crops ranged from 21.67-54.89 % (19). The variability in anthracnose disease incidence can be attributed to multiple contributing factors, such as continuous monocropping, conducive environmental conditions, agricultural practices, prolonged periods of high humidity followed by heavy rainfall, susceptibility of specific crop varieties and diverse cropping systems (16, 19). Additionally, leaf disease in groundnut crops has been reported to cause significant yield loss in Cuddalore, Tamil Nadu, using a deep learning based detection system (20, 21).

Moreover, the study on the cultural characteristics of various isolates of *Colletotrichum* spp. revealed that the colony colour ranged from fluffy white to greyish white and grey (Fig. 2). The fungal isolates collected from various locations exhibited distinct morphological characteristics. Notably, isolates Cg1 and Cg3 developed a moderately fluffy, white mycelial texture, whereas Cg2 and Cg9 displayed a dense, cottony white mycelium interspersed with vibrant orange conidial masses arranged in concentric rings. In contrast, isolates Cg5 and Cg8 demonstrated a greyish white mycelium, while isolates Cg4, Cg6 and Cg7 featured a combination of white, fluffy and partially submerged mycelial growth, highlighting the diversity in colony morphology among the isolates. Additionally, isolate Cg10 was characterized by cottony white mycelium with greyish conidial masses in concentric rings (Table 1). The maximum mycelial growth, measuring 90.00 mm, was observed in the Cg1 isolate (22), while the minimum mycelial growth of 45.70 mm was observed in isolate Cg5. It was noted that *Colletotrichum* spp.

Table 1. Survey, isolation and cultural characteristics of various isolates of *Colletotrichum* spp.

S. No.	Isolate name	Location	Crop stage	Variety	Disease incidence* (%)	Culture characteristics	Growth pattern	Mycelial growth* (mm)	Sporulation
1	Cg1	Sivapuri	Pod development	VRI 2	37.54^{a} (37.93)	Moderate fluffy white mycelium	Circular	90.00^{a} (72.20)	++++
2	Cg2	Parangipettai	Peg formation	ALR 3	14.22^{h} (22.15)	Greyish white cottony mycelium with orange conidial masses	Circular	61.00^{g} (51.35)	++
3	Cg3	Panruti	Pod development	TMVGn 13	27.78^{d} (31.80)	Moderate fluffy white mycelium	Wavy	80.56^{cd} (63.95)	++++
4	Cg4	Nallur	Pod formation	COGn 4	24.56^{e} (29.70)	Slightly white mycelium	Circular	76.32^{de} (60.92)	+++
5	Cg5	Kammapuram	Flowering	VRIGn 6	05.75^{j} (13.87)	Cottony greyish white mycelium	Circular	45.70^{i} (42.53)	+
6	Cg6	Bhuvanagiri	Peg penetration	VRI 3	34.73^{b} (36.10)	Fluffy white mycelium	Wavy	86.25^{b} (68.27)	++++
7	Cg7	Kurunjipadi	Pod development	VRIGn 6	17.94^{g} (25.05)	Submerged white mycelium	Circular	67.58^{f} (55.31)	+++
8	Cg8	Kattumannarkoil	Flowering	TMV 7	11.09^{i} (19.45)	Greyish white mycelium	Wavy	53.06^{h} (46.75)	+
9	Cg9	Keerapalayam	Pod formation	CO 3	30.69^{c}(33.63)	Fluffy cottony mycelium with orange conidial masses in concentric rings	Wavy	83.56^{bc} (66.17)	++++
10	Cg10	Virudhachalam	Flowering	VRI 2	20.48^{f} (26.90)	Cottony white with greyish conidial masses concentric rings	Circular	72.46^{ef} (58.19)	+++

++++ - Excellent, +++ - Good, ++ - Fair, + - Poor

*Mean of three replications

*In a column, mean followed by a common letter are not significantly differ at 5 % level by Ducan's multiple range test (DMRT).

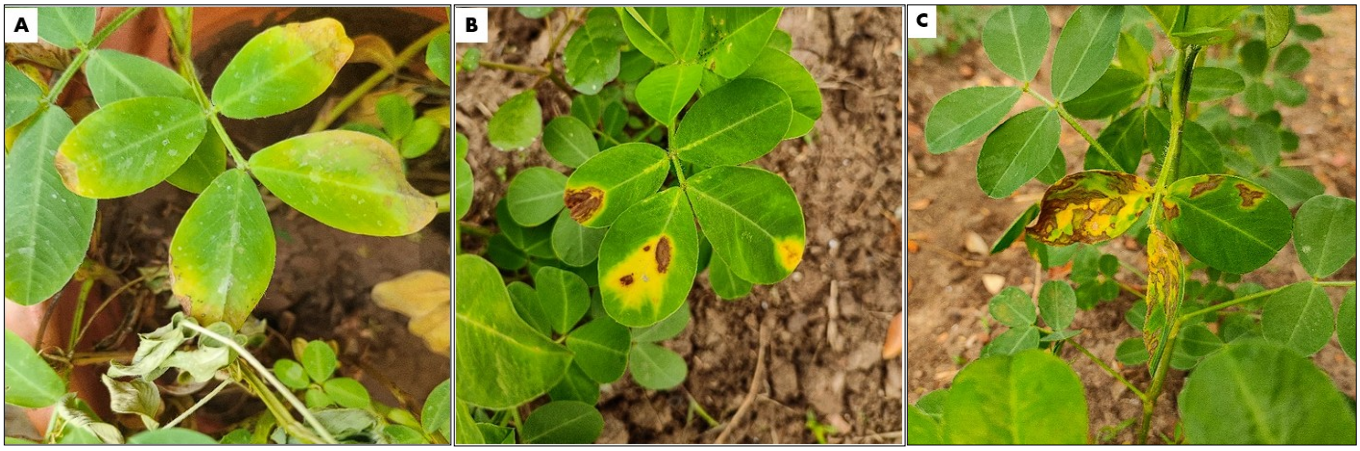

Fig. 1. Characteristic symptoms of anthracnose observed on groundnut leaf: A. Irregular marginal round lesions turning yellow to brown; B. Yellow to brown necrotic spots with water-soaked halos; lesions develop a concentric ring pattern as the disease progresses; C. Multiple dark brown necrotic spots merging to form larger lesions. Advanced infection causes leaf distortion and curling.

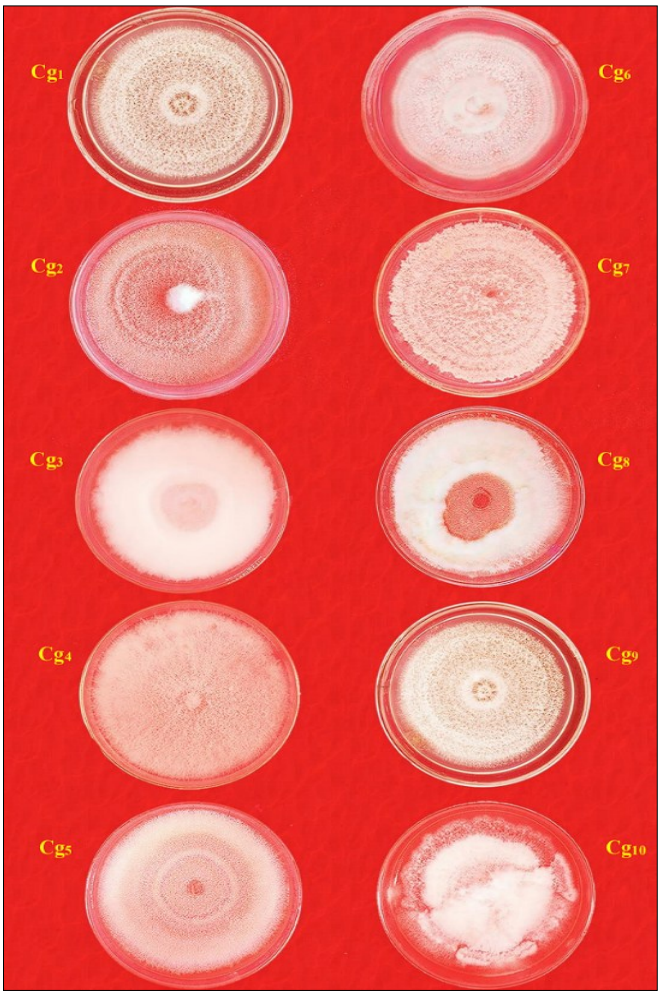

Fig. 2. Different isolates of *Colletotrichum* spp.

formed cottony greyish white mycelium with a circular cottony appearance on PDA medium and orange-coloured spots were observed upon culture maturation. Similar observations have been reported by earlier researchers (23-25).

Among the isolates, excellent sporulation was observed in Cg1, Cg3, Cg6 and Cg9, whereas good sporulation was recorded in Cg4, Cg7 and Cg10. Fair sporulation was noted in Cg2 and Cg5, while poor sporulation was observed in Cg8. The degree of sporulation was governed by distinct laboratory parameters and the nutritional profile of the culture medium. Additionally, variations in conidial colour and shape among the isolates were attributed to differences in their geographical origin and the utilization of nutritional factors for mycelial development, which played a significant role in determining the colour characteristics of *Colletotrichum* spp. (26).

Conidial characteristics

The conidial characteristics of the ten *Colletotrichum* spp. isolates exhibited considerable variation. Isolate Cg8 produced conidia with the maximum length and width of 16.55 × 6.13 µm, which were light brown in colour, fusiform in shape and had a conidial population of 0.7, followed by Cg1. In contrast, isolate Cg4 produced conidia with the minimum length and width of 10.48 × 3.92 µm, which were hyaline, cylindrical with rounded ends and had a conidial population of 1.9 (Table 2; Fig. 3, 4). The conidial shapes among the isolates ranged from round to cylindrical, cylindrical with round ends, elliptical and ellipsoidal. Consistent with the present study, the conidia of *C. gloeosporioides* have been reported to be unicellular, hyaline and cylindrical with round ends or ellipsoidal in shape (27). Similarly, conidia of *C. gloeosporioides* with sizes ranging from 10.00-12.33 × 3.00-4.33 µm have been documented (9, 28).

Pathogenicity test of various isolates of *Colletotrichum* spp. on the incidence of anthracnose on groundnut (var. VRI 2)

The disease incidence of ground nuts was evaluated to prove its pathogenicity. The findings from this study demonstrated significant variations in pathogenicity among different isolates. Among the ten *Colletotrichum* spp. isolates collected from major groundnut-growing regions of Cuddalore, the isolate Cg1, obtained from Sivapuri, exhibited the highest virulence. This strain recorded a maximum mean disease incidence of 40.57 % in pot culture experiments (Table 3; Fig. 5). The symptoms of *Colletotrichum* spp. in diseased groundnut plants initially appeared as marginal eyespots. These spots progressively enlarged at a rapid pace, becoming irregular in shape and eventually spreading across the entire leaflet, leading to extensive tissue damage. As the infection progressed, dark lesions would appear on the leaves, eventually forming a concentric ring pattern after 7th days after inoculation. The inoculation tests of various isolates of *C. gloeosporioides* had satisfied Koch's postulates (3). The plants could develop symptoms of anthracnose disease within 6 DAI (29). Several factors contribute to disease development and incidence, with high humidity and cool weather being significant predisposing conditions for anthracnose. Seasonal rainfall in November and December further supports inoculum potential and facilitates disease formation. Additionally, the presence of dew during this period serves as another contributing factor, promoting disease incidence (29).

Table 2. Conidial characteristics of various isolates of *Colletotrichum* spp.

S. No.	Isolate name	Length of conidia (µm)*	Breadth of conidia (µm)*	Conidial character				
				Shape of conidia	Conidia colour	Acervuli	Setae	Conidial population × 10^6/ mL
1	Cg1	14.55	05.17	Elliptical	Hyaline	Present	Present	2.9
2	Cg2	12.42	03.75	Cylindrical	Hyaline	Present	Present	0.9
3	Cg3	11.28	03.07	Curved with round apex	Hyaline, colourless	Absent	Absent	2.1
4	Cg4	10.48	03.92	Cylindrical with round end	Hyaline	Present	Present	1.9
5	Cg5	11.25	03.69	Cylindrical	Hyaline	Absent	Absent	0.6
6	Cg6	13.34	04.23	Ellipsoidal	Hyaline	Present	Present	2.5
7	Cg7	11.82	02.75	Oval to cylindrical with truncate base	Hyaline	Present	Present	1.2
8	Cg8	16.55	06.13	Fusiform	Light brown	Absent	Absent	0.7
9	Cg9	11.46	06.78	Ellipsoidal	Hyaline	Present	Present	2.4
10	Cg10	12.06	04.72	Cylindrical	Hyaline, colourless	Absent	Absent	1.5

*Mean of three replications

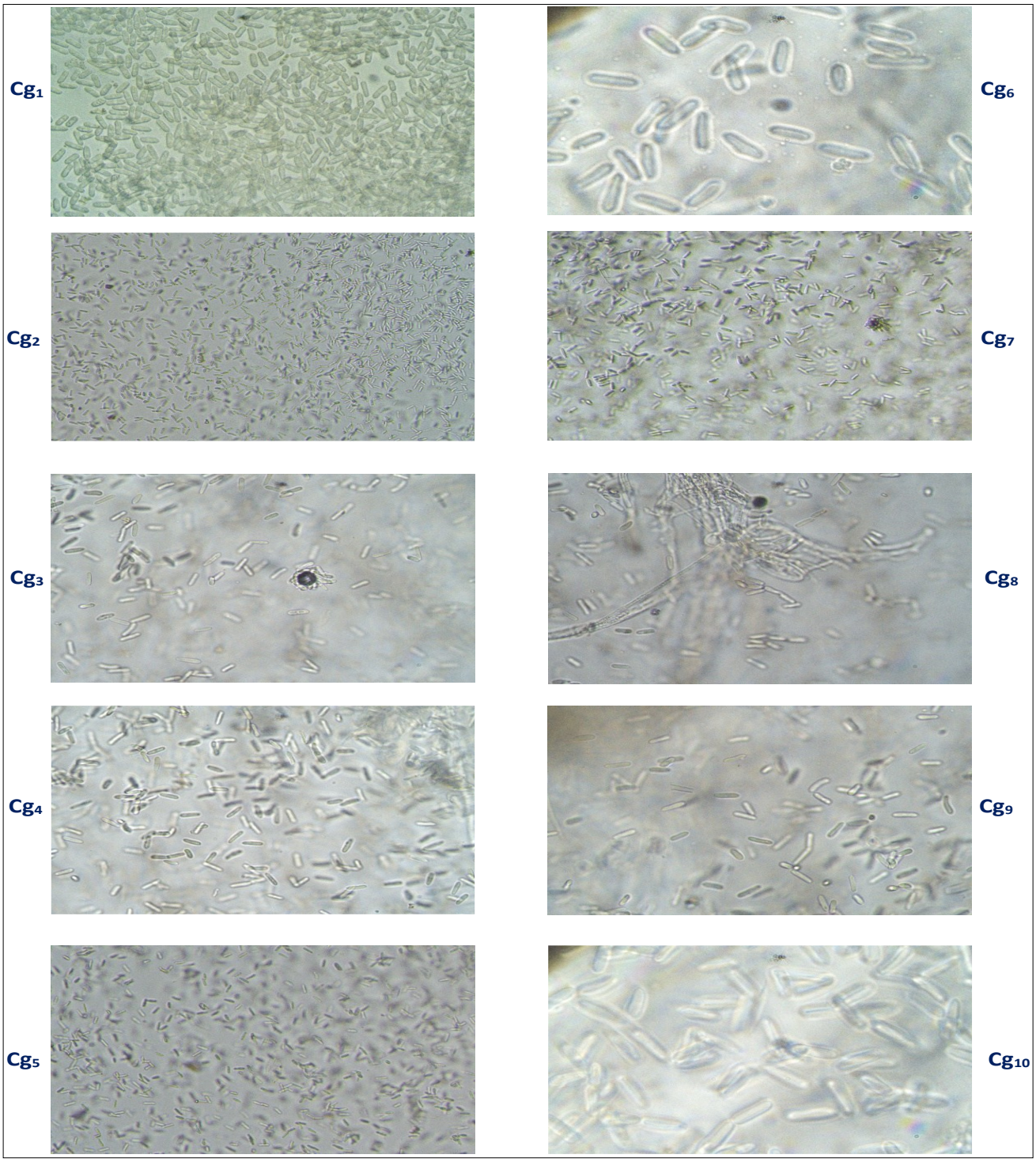

Fig. 3. Microscopic image of various isolates of *Colletotrichum* spp.

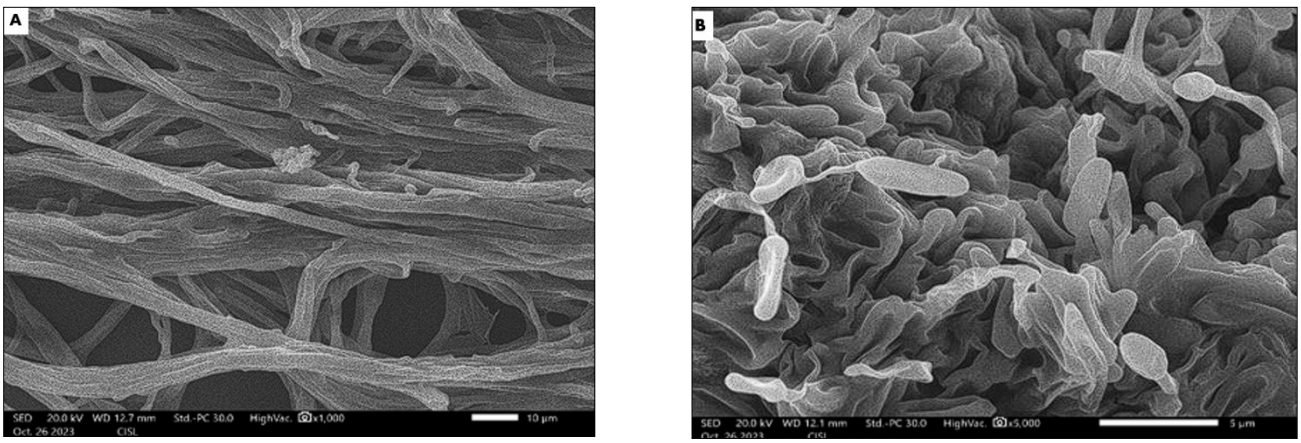

Fig. 4. SEM image of *Colletotrichum* spp.: A. Aggregation of mycelial threads of *Colletotrichum* spp.; B. Conidial spore of *Colletotrichum* spp.

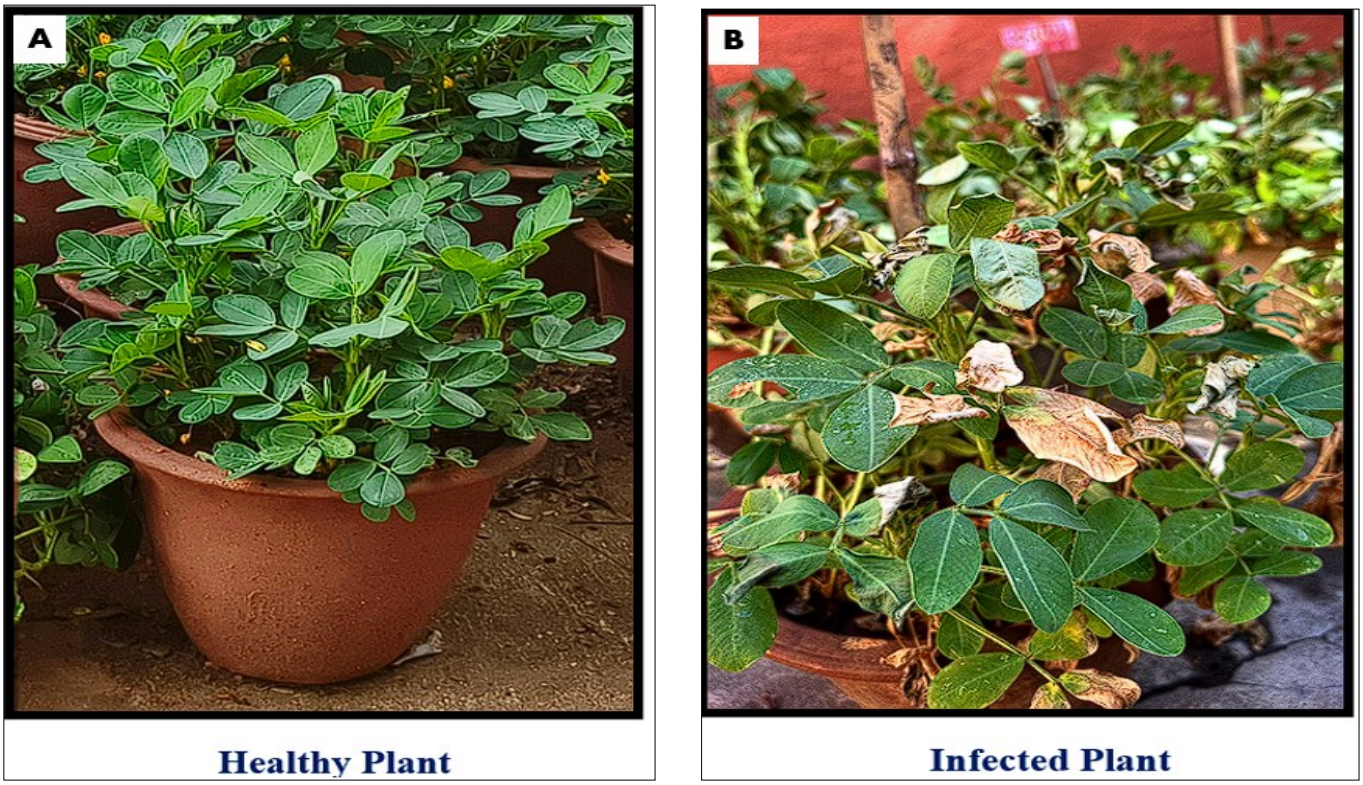

Fig. 5. Pathogenicity test of *Colletotrichum* spp.: A. Negative control; B. Brown lesions followed by a blighted appearance induced by *Colletotrichum* spp. (positive control).

Molecular characterization of virulent isolate

The ITS region of *Colletotrichum* isolates was amplified using the previously established primer pair ITS1-F and ITS4-R, generating a distinct DNA fragment approximately 550 bp in length. Among the ten isolates analyzed, the ITS sequencing of the most aggressive strain, designated as Cg1, conclusively placed it within the *C. gloeosporioides* species complex. A BLAST analysis conducted on the NCBI database, confirmed its identity, aligning with the GenBank sequence MG204864.1. The obtained sequence was later submitted to GenBank and assigned the accession number OR414782.1. To further elucidate the phylogenetic relationships, a similarity matrix was constructed and a robust phylogenetic tree was constructed using the MEGA X 11 software, reinforcing the classification of Cg1 as the most virulent isolate subjected to molecular characterization (Fig. 6, 7). Similarly, researchers amplified the 5.8S-ITS region of *Colletotrichum* spp. using the universal primers ITS1 and ITS4 (30). It has been reported that amplification of the ITS1-5.8S-ITS2 region of rDNA using these primers produces a fragment of approximately 550 bp for *C. gloeosporioides* isolates. All *Colletotrichum* isolates in the study yielded the expected amplicon length of 550 bp when amplified with ITS1 and ITS4 primers (31).

Physiological investigations on mycelial growth of *C. gloeosporioides* (Cg1)

Impact of various solid and liquid culture media

Among the various solid media (Fig. 8) evaluated for their effectiveness in supporting the growth and sporulation of *C. gloeosporioides* (Cg1), PDA exhibited the highest mycelial expansion, reaching 87.39 mm. This was closely followed by Czapek's Dox Agar, which supported a mycelial growth of 82.63 mm. It has been reported that *Collectotrichum* spp. grow well in PDA compared to other media (32). The present study also revealed excellent sporulation on PDA medium, with superior sporulation on carrot agar and potato sucrose agar (Fig. 9). Mycelial growth of *C. gloeosporioides* was luxuriant in PDA medium, followed by Czapek's Dox Agar. The luxuriant growth observed in PDA may be attributed to its rich nutrient content, particularly dextrose, which promotes mycelial development and sporulation in the medium (33).

Table 3. Pathogenicity test of various isolates of *Colletotrichum* spp. on the incidence of anthracnose on groundnut (var. VRI 2)

S. No.	Isolate name	Disease incidence (%)			Mean*
		40 DAS*	65 DAS*	90 DAS*	
1	Cg1	29.40	44.85	47.47	40.57^{a} (39.72)
2	Cg2	08.96	18.78	20.11	15.95^{h} (23.53)
3	Cg3	12.39	30.53	32.94	25.29^{d} (30.18)
4	Cg4	13.74	24.16	26.57	21.49^{e} (27.61)
5	Cg5	04.11	11.71	14.46	10.09^{j} (18.51)
6	Cg6	17.13	41.73	44.36	34.41^{b} (35.91)
7	Cg7	10.25	20.44	23.23	17.97^{g} (25.07)
8	Cg8	06.37	13.39	15.89	11.88^{i} (20.16)
9	Cg9	15.97	36.42	38.05	30.15^{c} (33.30)
10	Cg10	11.62	21.55	24.69	19.29^{f} (26.00)

*Mean of three replications

*In a column, mean followed by a common letter are not significantly differ at 5 % level by Ducan's multiple range test (DMRT).

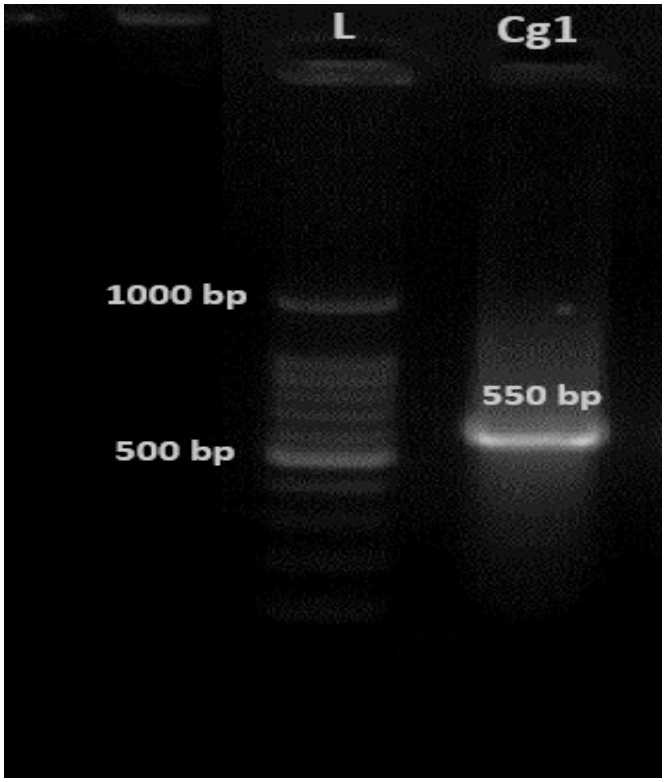

Fig. 6. Agarose (1.5 %) gel electrophoresis of dsDNA extracted from the *C. gloeosporioides*: Amplification product obtains from PCR. Lane L: DNA ladder with reference bands at 1000 bp and 500 bp; Lane Cg1: *C. gloeosporioides* (Accession No- OR414782.1) showing a 550 bp amplicon, confirming successful amplification of the target gene.

The assessment of various liquid media (Fig. 10) for their influence regarding the fungal biomass accumulation of the pathogen (Table 4) demonstrated that Potato Dextrose Broth facilitated the highest biomass accumulation, recording a mycelial dry weight of 329.78 mg. This was closely followed by Czapek's Dox Broth, which yielded a substantial dry weight of 284.36 mg (Fig. 11). Potato Dextrose Broth ranked highest in supporting mycelial growth due to its rich nutrient content, dextrose and abundant carbon sources, which facilitate fungal development and mycelial formation (16).

Influence of various light regimes

C. gloeosporioides was exposed to the various light regimes to test the fungal development. Isolate Cg1was grown on five different light regimes (Table 5) and among them, isolate Cg1 grown luxuriantly on 12 hr of light and 12 hr of dark period. In agreement with the present study researchers observed that incubation of *C. gloeosporioides* under a 12 hr of light and 12 hr of dark cycle resulted in markedly superior colony growth compared to other treatments (Fig. 12, 13) (16).

It was observed that under a 12 hr of light and 12 hr of dark cycle, the pathogen *Colletotrichum capsici* exhibited enhanced mycelial development and conidial sporulation, thereby improving the physiological growth of the fungus. However, when the culture was placed under continuous darkness (24 hr dark), it yielded the least colony growth of the target test fungus (34). Many researchers have reported that colony development needed 12 hr of light and 12 hr of dark. The crucial factor for conidia development was identified by observing a 12 hr of light and 12 hr of dark cycle, which served as the key stimulating factor for conidia formation and acervuli development *in vitro* (35).

Influence of various temperature and pH

In this study, the mycelial expansion of isolate Cg1 was assessed across six distinct temperature regimes (Fig. 14, 15) and pH gradients (Fig. 16, 17). The optimal growth was recorded at 30 ºC and pH 5.0, exhibiting a radial extension of 87.53 mm and 80.37 mm respectively, with corresponding dry biomass yields of 306.98 mg and 177.67 mg. Conversely, the most restricted growth was observed at 40 ºC and pH 10.0, measuring only 12.69 mm and 56.70 mm, with dry biomass weights of 21.13 mg and 56.70 mg respectively (Table 6). The fungal pathogen *C. gloeosporioides* demonstrated peak colony expansion at pH 5.0, which showed a progressive decline as the medium's pH increased. The second-highest colony growth was recorded at pH 6.0, followed by moderate expansion at pH 7.0, while pH 8.0 exhibited the lowest colony proliferation, further diminishing at pH 9.0 (16). Observations also revealed that the maximum mycelial proliferation and conidial germination occurred at 25 °C, followed by slightly lower mycelial proliferation and conidial germination at 30 °C (17, 35).

The key factors influencing the *in vitro* and *in vivo* development of mycelia and conidia were temperature, light and pH. The optimal temperature range for proliferation was recorded between 25 °C and 30 °C (36). Light plays a crucial role in the formation and development of *Colletotrichum* mycelia and spores, significantly impacting conidia and mycelial formation. Additionally, pH was an important determinant, with an ideal pH of around 5 being optimal for mycelial mat and conidia development (35).

Table 4. Effect of different culture media on the growth characteristics and dry biomass weight of *C. gloeosporioides* (Cg1)

S. No.	Solid medium	Culture characteristics	Mycelial growth* (mm)	Sporulation	Liquid medium	Dry biomass weight* (mg)
1	Corn Meal Agar	Pale white mycelium	16.91^{g}	+	Corn Meal Broth	79.45^{g}
2	Czapek's Dox Agar	Fluffy light pink mycelium	82.63^{b}	++++	Czapek's Dox broth	284.36^{b}
3	Potato Sucrose Agar	Greyish white mycelium	70.25^{d}	+++	Potato Sucrose Broth	178.53^{d}
4	Beetroot Agar	Pale red to white mycelium	45.74^{e}	++	Beetroot Broth	132.15^{e}
5	Potato Dextrose Agar	Moderate fluffy white mycelium	87.39^{a}	++++	Potato Dextrose Broth	329.78^{a}
6	Carrot Agar	Cottony white mycelium	75.18^{c}	+++	Carrot Broth	227.61^{c}
7	Oat Meal Agar	Slightly white mycelium	24.32^{f}	+	Oat Meal Broth	92.13^{f}

++++ - Excellent, +++ - Good, ++ - Fair, + - Poor

*Mean of three replications

*In a column, mean followed by a common letter are not significantly differ at 5 % level by Ducan's multiple range test (DMRT).

Table 5. Influence of light regimes on the mycelial growth and sporulation of *C.gloeosporioides* (Cg1)

S. No.	Light regimes	Colony growth* (mm)			Sporulation on 9th DAI
		4th DAI	6th DAI	8th DAI	
1	24 hr light	25.83[d]	38.64[d]	58.28[d]	+++
2	24 hr dark	21.49[e]	32.95[e]	51.16[e]	+
3	12 hr light, 12 hr dark	39.58[a]	63.22[a]	82.61[a]	++++
4	16 hr light, 8 hr dark	32.67[b]	47.38[b]	65.53[b]	++
5	8 hr light, 16 hr dark	27.88[c]	40.26[c]	60.76[c]	++

++++ - Excellent, +++ - Good, ++ - Fair, + - Poor

DAI- Days after inoculation

*Mean of three replications

*In a column, mean followed by a common letter are not significantly differ at 5 % level by Ducan's multiple range test (DMRT).

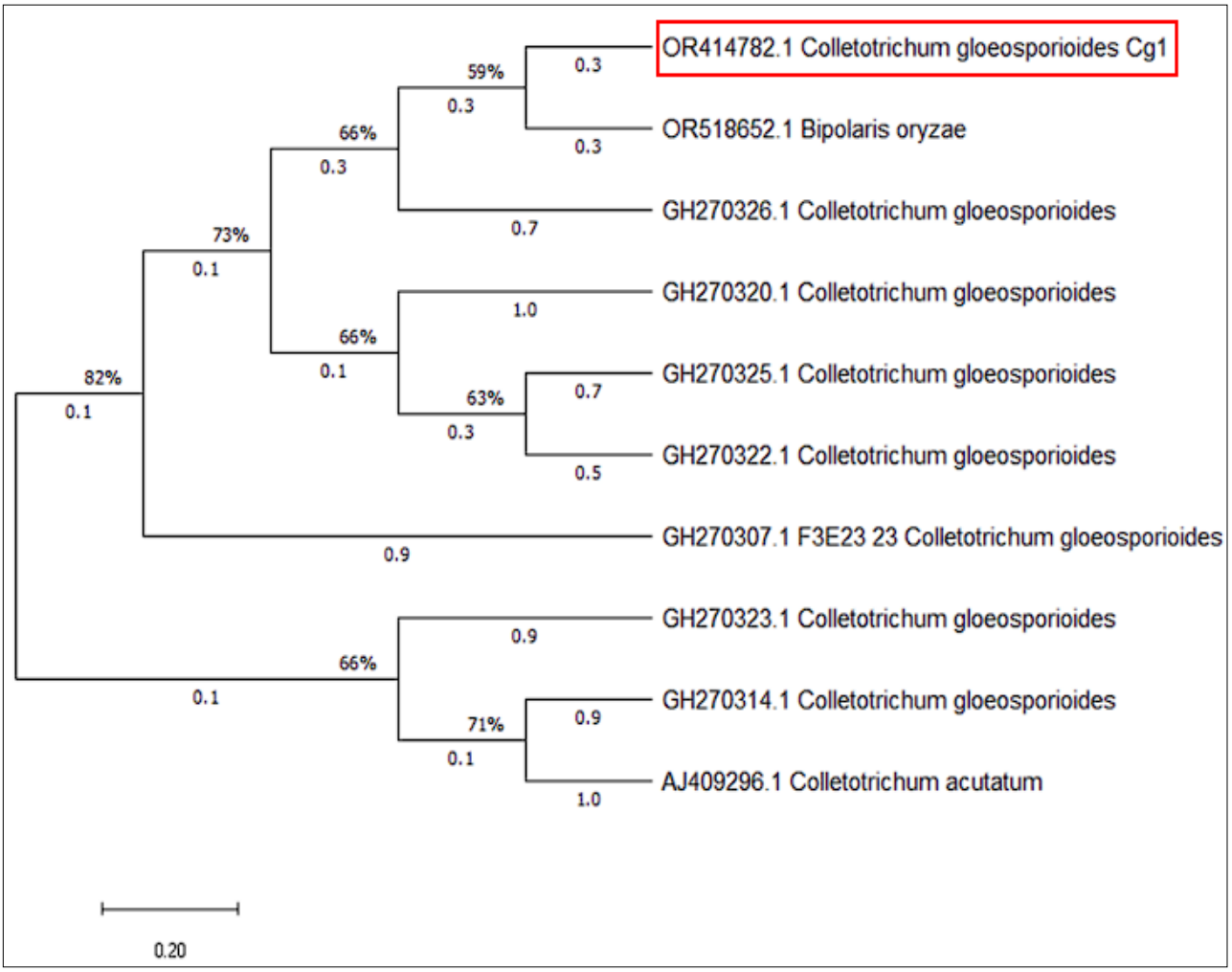

Fig. 7. Phylogenetic tree of *C. gloeosporioides* species complex (Cg1) isolate.

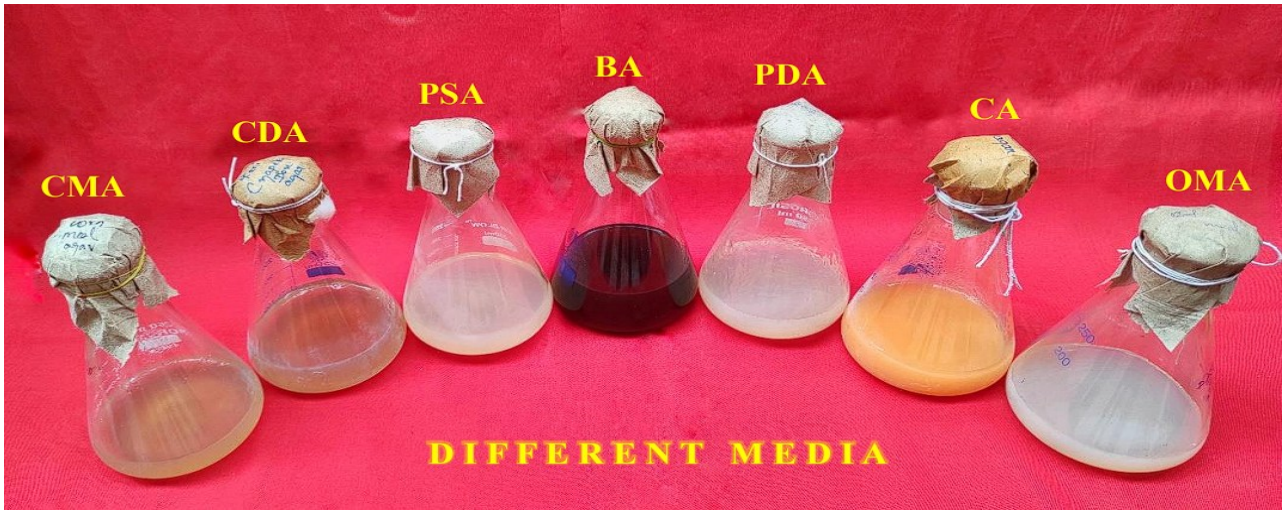

Fig. 8. Different solid media: CMA - Corn Meal Agar; BA - Beetroot Agar; CDA - Czapek's Dox Agar; PDA - Potato Dextrose Agar; OMA- Oat Meal Agar; PSA- Potato Sucrose Agar; CA - Carrot Agar.

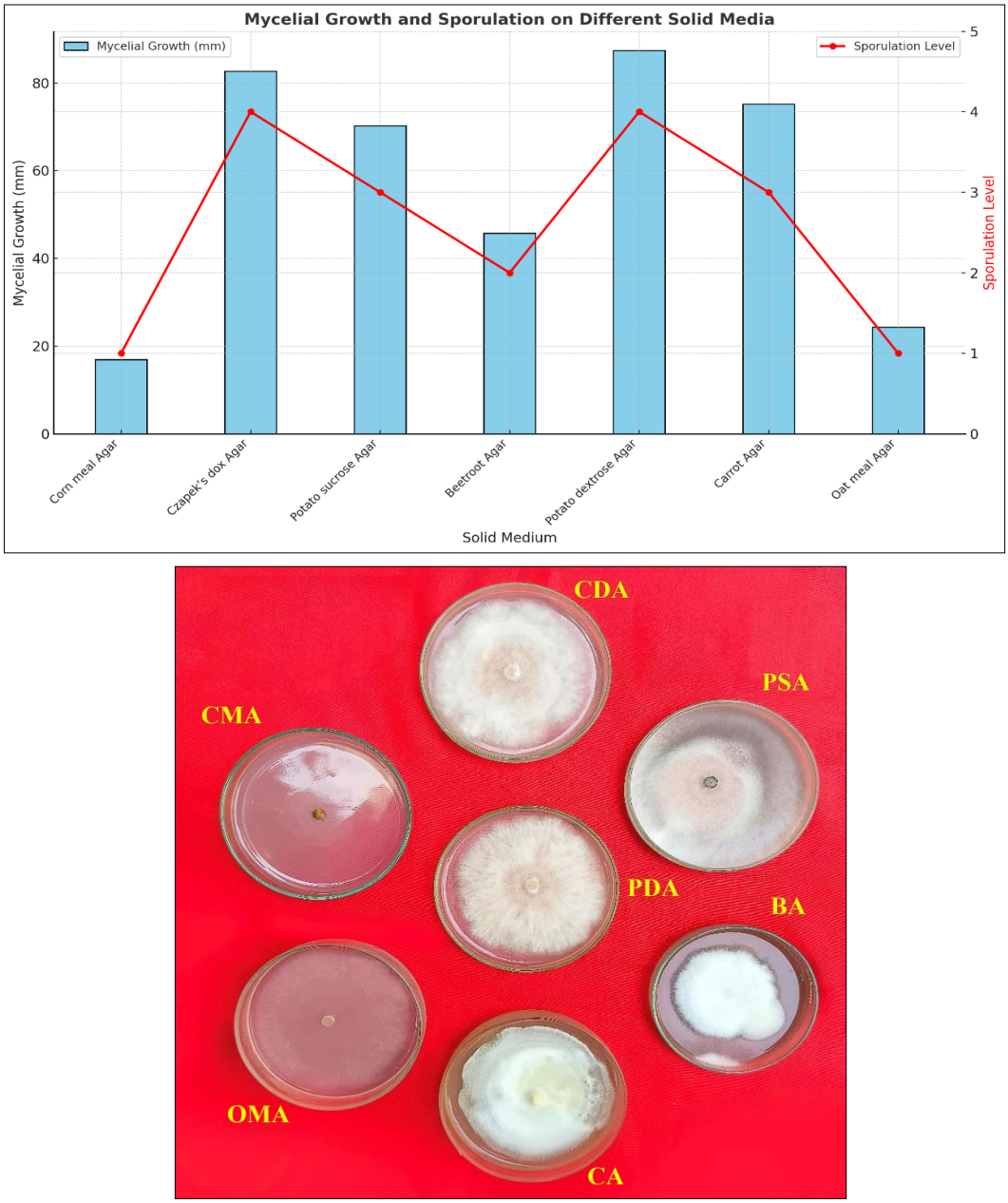

Fig. 9. Mycelial growth and sporulation on different solid media: A. Data visualization; B. Culture plates.

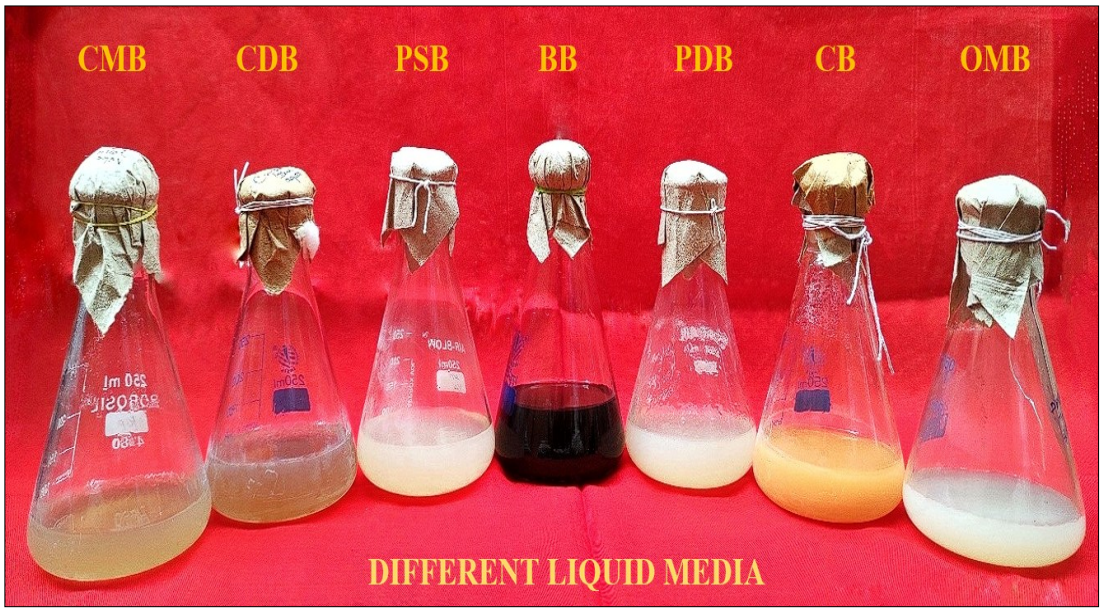

Fig. 10. Different liquid media: CMB- Corn Meal Broth; BB- Beetroot Broth; CDB- Czapek's Dox Broth; PDB- Potato Dextrose Broth; PSB- Potato Sucrose Broth; CB- Carrot Broth; OMB- Oat Meal Broth.

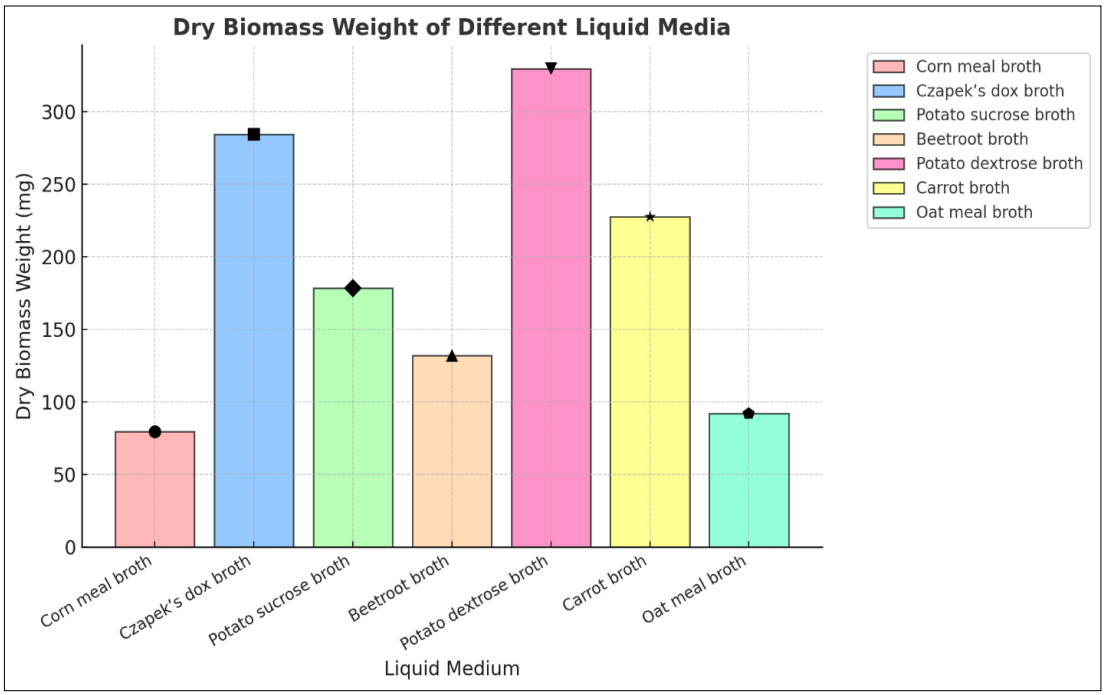

Fig. 11. Mycelial growth and sporulation on different liquid media.

Fig. 12. Growth of *C. gloeosporioides* on different light regimes.

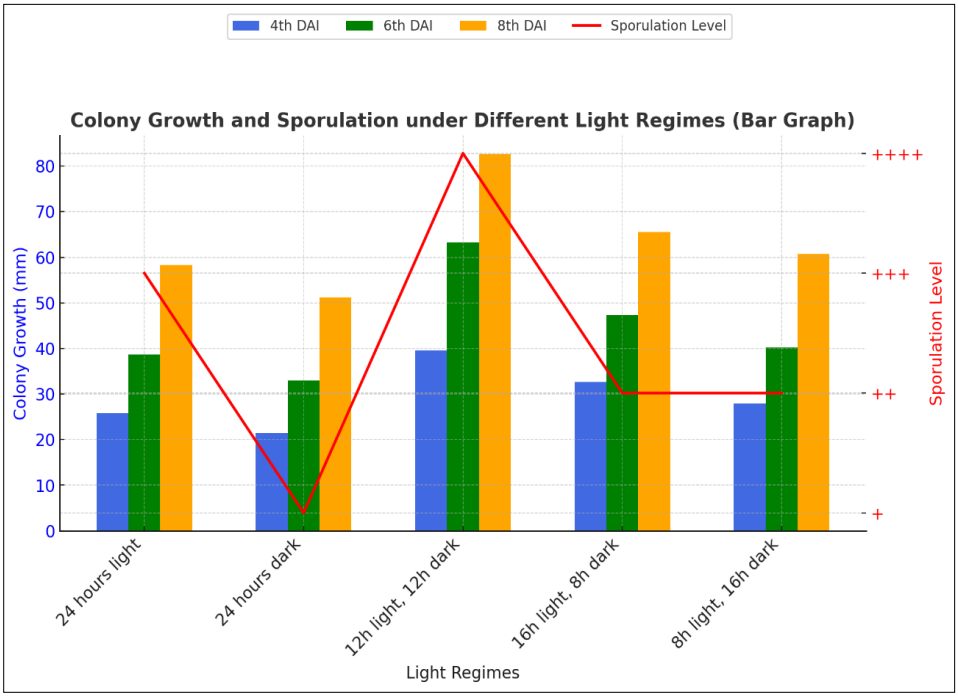

Fig. 13. Colony growth and sporulation under different light regimes.

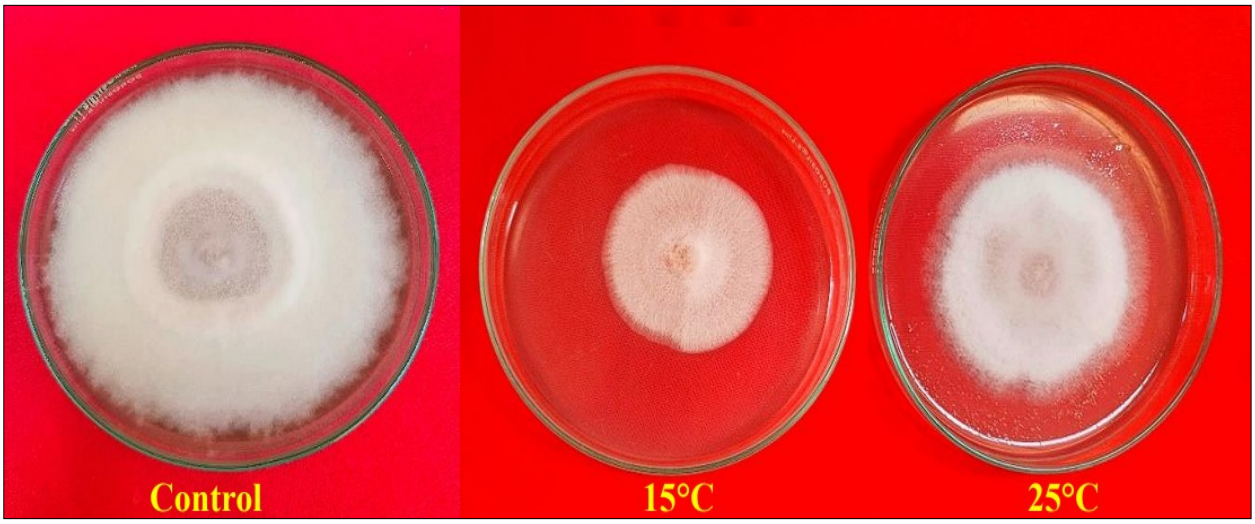

Fig. 14. Growth of *C. gloeosporioides* on different temperature.

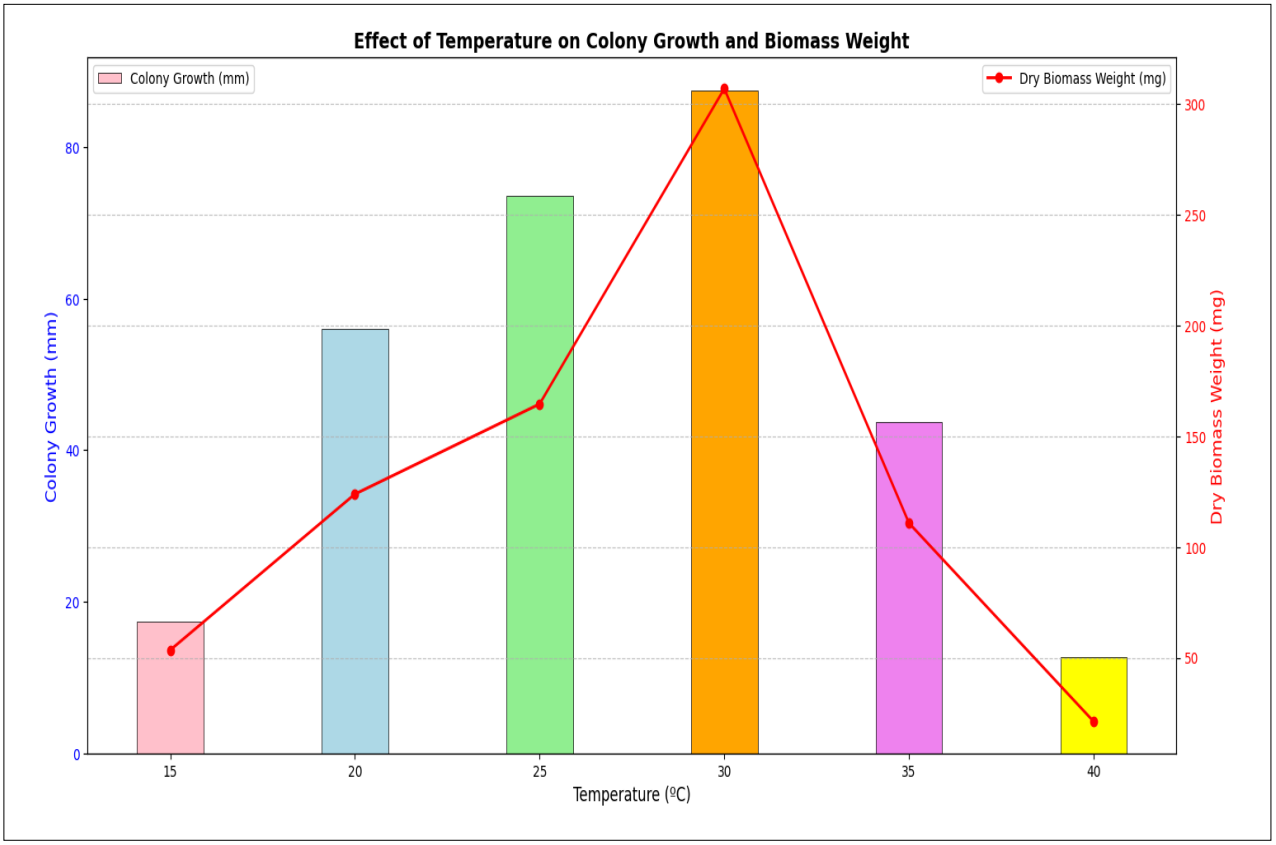

Fig. 15. Effect of temperature on colony growth and biomass weight.

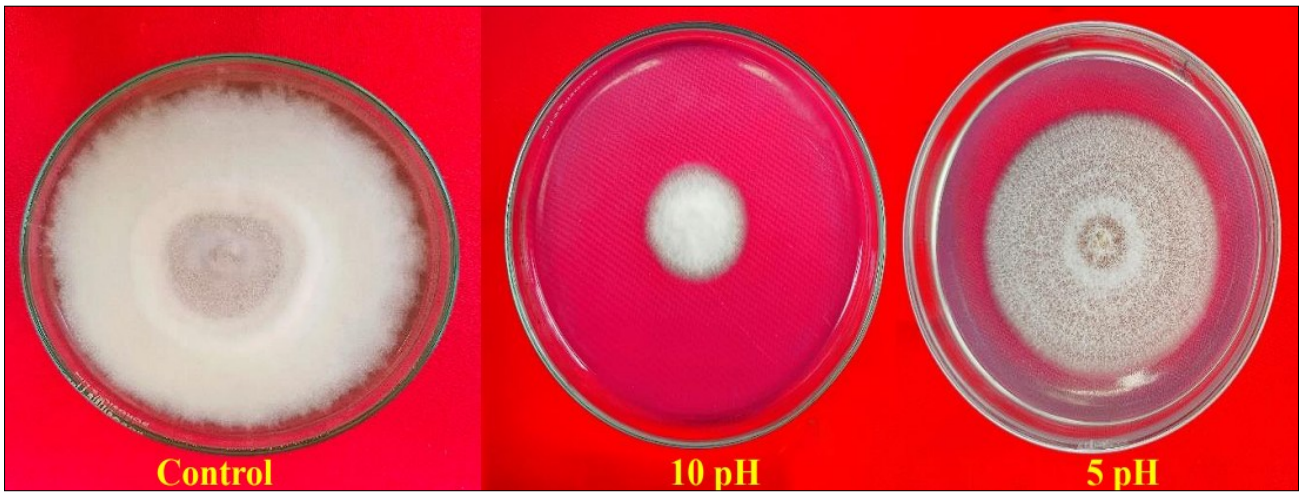

Fig. 16. Growth of *C. gloeosporioides* on different pH.

Fig. 17. Effect of pH on colony growth and biomass weight.

Table 6. Effect of different temperature and pH level on the mycelial growth and dry biomass weight of *C. gloeosporioides* (Cg1)

S. No.	Different temperature				Different pH level			
	Temperature (°C)	Colony growth* (mm)	Dry biomass weight* (mg)	Sporulation	pH level	Colony growth* (mm)	Dry biomass weight* (mg)	Sporulation
1	15	17.31^{e}	53.43^{e}	+	5.0	80.37^{a}	177.67^{a}	++++
2	20	55.98^{c}	123.86^{c}	++	6.0	76.63^{b}	162.91^{b}	++++
3	25	73.64^{b}	164.55^{b}	+++	7.0	54.32^{c}	143.52^{c}	+++
4	30	87.53^{a}	306.98^{a}	++++	8.0	47.16^{d}	119.43^{d}	++
5	35	43.72^{d}	110.75^{d}	+	9.0	39.54^{e}	92.05^{e}	+
6	40	12.69^{f}	21.13^{f}	+	10.0	27.05^{f}	56.70^{f}	+

++++ - Excellent, +++ - Good, ++ - Fair, + - Poor

*Mean of three replications

*In a column, mean followed by a common letter are not significantly differ at 5 % level by Ducan's multiple range test (DMRT).

Conclusion

In light of the present investigation, the variability in disease incidence across different locations and cultivars is influenced by factors such as environmental conditions, cultivation practices and monocropping. Detailed cultural, physiological and molecular characterization of isolates, particularly Cg1 from Sivapuri, revealed its virulence and optimal growth conditions. Based on existing literature, this study presents the first report from Tamil Nadu, India, identifying *C. gloeosporioides* as a significant threat to groundnut cultivation in the region. These findings underscore the need to understand pathogen dynamics and environmental influences to develop effective management strategies.

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Authors' contributions

MSS designed the study, conducted molecular genetic analyses, participated in sequence alignment and drafted the manuscript. LV and TM conceptualized the idea and performed the statistical analysis. SS and AM conceived the study and contributed to its design and coordination. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interest to declare.

Ethical issues: None

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