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# Unmasking the Menace: Characterisation of *Alternaria alternata* Associated with Aloe Vera Leaf Spot in Karnataka, India

M. Muhammad Suhaib Ismayil, Y. M. Somasekhara, Farooqkhan, K. B. Palanna, H. A. Prameela and B. S. Chethana
Department of Plant Pathology, College of Agriculture, UAS, GKVK, Bengaluru - 560 065
e-Mail : vivaravinimayam@gmail.com

**Authors Contribution**

M. Muhammad Suhaib Ismayil :
Investigation, orginal draft prepration and analysis;
Y. M. Somasekhara :
Conceptualization, framing of research proposal, guidence and orginal draft correction;
Farooqkhan, K. B. Palanna, H. A. Prameela and B. S. Chethana :
Guidence and manuscript correction

***Corresponding Author*** **:**
M. Muhammad Suhaib

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Abstract

Aloe vera (*Aloe barbadensis*) is a medicinal plant of great economic importance due to its extensive use in pharmacy and cosmetics. However, the emergence of leaf spots substantially threatens its cultivation and reduces market value. This research comprehensively characterised *Alternaria alternata* responsible for Aloe vera leaf spot in Karnataka, India. Sampling from the Gandhi Krishi Vigyan Kendra (GKVK) in Bengaluru revealed distinctive leaf spot symptoms. Following isolating *Alternaria*-like colonies from infected Aloe vera leaves, the patho genicity assay was conducted to establish Koch's postulates, which induced characteristic lesions akin to field observations. Morphological examination highlighted traits consistent with *Alternaria*'s previously reported features. Molecular characterisation through ITS sequencing and NCBI-BLAST analysis confirmed the pathogen as *Alternaria alternata*. In-depth cultural characterisation of different media unveiled varied growth patterns, with Richard's Synthetic Agar and Oatmeal Agar promoting maximal radial mycelial growth. Physiological studies revealed optimal growth conditions for *Alternaria alternata*: temperatures between 25-30 °C and a pH range of 6-7. This study contributes vital insights into the identification and characterisation of *Alternaria alternata* associated with aloe vera leaf spots.

*Keywords* : Aloe vera, *A. alternata*, Morphological, Molecular, Cultural & Physiological characterisation

*Aloe barbadensis* Miller (aloe vera) is a succulent medicinal perennial herb, a member of the *Asphodelaceae* (formerly a member of the *Liliaceae*) family (Ozsoy *et al*., 2009). The aloe vera plant, renowned for its therapeutic and cosmetic attributes, has become an agricultural mainstay and a significant source of income for farmers. Aloe vera gel consists of about 98.5-99.5 per cent water and the remaining solid portion, contains more than 200 different components (Femenia *et al*., 1999). Among them, polysaccharides and phenolic compounds are the main bioactive components that impart therapeutic properties to the plant (Guo & Mei, 2016; Minjares-Fuentes *et al*., 2016 and Pothuraju *et al*., 2016). However, the cultivation of aloe vera is plagued by various challenges and one of the most pressing issues is the emergence of leaf spot disease caused by the fungal pathogen *Alternaria alternata*.

*Alternaria alternata* causes characteristic lesions on aloe vera leaves, which results in reduced plant vitality and decreased market value. *Alternaria* is a well-known phytopathogen with a broad host range; Moreover, the warm, humid climatic conditions favour its prevalence. The comprehensive research on its cultural, morphological and molecular characteristics concerning aloe vera is notably limited. To control the disease, it is essential to characterise the pathogen.



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Therefore, this study addresses the critical knowledge gap by providing a comprehensive characterisation of *Alternaria alternata* responsible for aloe vera leaf spots.

## Material and Methods

### Sample Collection, Fungal Isolation and Pathogenicity Assay

In May 2022, aloe vera leaves showing leaf spot symptoms were observed at the medicinal garden of the Gandhi Krishi Vigyan Kendra (GKVK) (13°05'N, 77°34'E), Bengaluru, India. Leaves having leaf spot symptoms were then collected. Percentage Disease Incidence (PDI) was determined using the following equation:

$$\text{PDI (\%)} = \frac{\text{Number of infected plants}}{\text{Total number of plants}} \times 100$$

Initially, symptomatic lesions were examined under a compound microscope (specifically, a Leica DM4B upright microscope) to identify fungal structures such as conidia and conidiophores. Following standard phytopathological protocols, the pathogen was subsequently isolated from these symptomatic lesions. In short, tiny sections of leaves containing both symptomatic and asymptomatic areas were sliced into pieces measuring 5x5 mm. They were then surface sterilised by immersing them in a 1 per cent sodium hypochlorite solution for 3 to 5 minutes, then rinsing with sterile distilled water. Afterwards, these sections were air-dried on blotting paper, placed onto a Petri dish containing PDA (Potato Dextrose Agar) and kept in the dark at a temperature of 25°C for a duration of one week. The pure culture was obtained using the single spore isolation technique (Zhang *et al*., 2013).

Pathogenicity assessments for the pathogen were conducted by spraying the homogeneous spore suspension. First, we utilised a modified slide culture technique to stimulate ample sporulation. Subsequently, we gently washed the glass slide to collect conidia using sterile distilled water (SDW). The resulting conidial suspension was then meticulously adjusted to a concentration of $2 \times 10^6$ conidia/ml with the aid of a haemocytometer, following Abkhoo *et al*. (2014). Next, by employing a hand atomiser, we uniformly applied this well-mixed suspension onto the pin-pricked leaves of aloe vera plants aged 60 days. For control purposes, another group of plants received a spray of sterile distilled water. All these plants were enveloped in transparent plastic bags and placed within an artificial inoculation chamber maintained at a temperature of 28°C and a relative humidity of 80 per cent for a duration of 21 days. Throughout this period, we diligently observed the plants for any indications of symptom onset and progression. It is important to note that we conducted all these procedures in triplicate to ensure the reliability of our results. Finally, we verified Koch's postulates by re-isolating the pathogen and confirming its identity by examining micro and macro-morphological characteristics.

### Morphological Characterization

The colony was examined at various stages of growth, ranging from 1 to 4 weeks old cultures. Microscopic analysis of the micro-morphology was conducted using a modified slide culture technique. To create the modified slide culture unit, microscopic glass slides were arranged within a Petri dish with filter paper discs positioned at the top and bottom to maintain the necessary moisture for fungal growth. This entire unit was sterilised through autoclaving. Mycelial fragments were placed on the glass slide using a sterile inoculation needle and the germination paper was moistened using sterile water from a sterile pipette. Subsequently, the unit was incubated for three days, after which the slide was removed and mounts were prepared for further microscopic examination. The morphological characters, such as the size and shape of the conidia and the number of transverse and longitudinal septa were recorded.

### Molecular Characterisation

### DNA Extraction, Polymerase Chain Reaction (PCR) Amplification and Sequencing

Ten-day-old fungal mycelium from a culture grown on PDB was harvested. The genomic DNA of isolat0e

was extracted using the CTAB protocol (Murray and Thompson, 1980) with minor modifications. PCR amplification was performed on universal fungal genomic loci ITS. Amplification was carried out in 20 µL reaction mixtures containing 2 µL DNA template, 1 µL of each primer, 6 µL of double-distilled water (ddH2O) and 10 µL of master mix (TAKARA®). The PCR products were purified using a gel extraction kit (Medauxin®) following the manufacturer's protocol and sequenced at Eurofins (Bengaluru, India).

**Cultural Characterization of *Alternaria alternata* on different Solid Media**

The cultural characters of *Alternaria alternata* such as colony diameter, colony form and elevation, colony margin, zonation, colony colour, pigmentation and sporulation were studied on ten different media *viz*., Corn Meal Agar (CMA), Malt Extract Agar (MEA), Rose Bengal Agar (RBA), Czapek Dox Agar (CDA), Oat Meal Agar (OMA), Potato Carrot Agar (PCA), Potato Dextrose Agar (PDA), Sabouraud Dextrose Agar (SDA), Richard's Synthetic Agar (RSA) and V8 Juice Agar (VJA). The composition and preparation of the above-mentioned media were obtained from Ainsworth and Bisby's 'Dictionary of the Fungi' by Ainsworth (1971), Plant Pathological Methods, Fungi and Bacteria by Tuite (1969). All the ingredients were dissolved in 400 ml of distilled water and agar was dissolved separately in 500 ml of distilled water and mixed with the above solution and the volume was made up to 1000 ml. The medium was sterilised at 1.1 kg/cm$^2$ pressure for 20 min.

Fifteen mL of each medium listed above was poured into Petri plates of 90 mm diameter and allowed to solidify. Such plates were inoculated with 5 mm discs from 10-days-old cultures of *Alternaria alternata*, which were cut using a cork borer and a single disc was placed upside down at the centre of the plate. Each set was replicated thrice and the plates were incubated at 28 ± 1°C. Observations were recorded when the maximum growth was attained in all the media tested. Using a transparent plastic scale, the linear growth of the colony was measured in millimetres. In addition, the sporulation was observed from 14-day-old culture by making the spore suspension. A single block of 5 mm diameter was cut out from the fungal colony near the margin by sterilised cork borer. It was transferred to 5 ml sterile distilled water in a test tube and mixed thoroughly to make a uniform spore suspension. One small drop of spore suspension was taken on a slide. Following, the average spore count of three microscopic fields under the microscope's low power (10X) objective was estimated. The sporulation was graded as follows.

| Sore | Grade | Conidia/ microscopic field (100x)* |
|---|---|---|
| ++++ | Excellent | >40 |
| +++ | Good | 21-40 |
| ++ | Fair | 11-20 |
| + | Poor | 1-10 |
| - | No sporulation | - |

* Mean (n = 20) observations under 100 x magnification

**Physiological Studies**

**Effect of Temperature on Growth of *Alternaria alternata***

The growth of the fungus was tested at 15°C, 20°C, 25°C, 30°C and 35°C. 100 ml flasks containing 50 ml of sterilised potato dextrose broth were inoculated with a 5 mm disc of the pathogen. Simultaneously, Petri plates containing sterilised 15 ml of potato dextrose agar medium were inoculated with 5 mm discs of the pathogen. The conical flasks and Petri plates were then incubated at five temperatures for two weeks and one week, respectively. The experiment was conducted using a Completely Randomized Design (CRD) and each treatment was replicated four times. The mycelial mat in PDB was harvested by filtering through Whatman No.1 filter paper and dried in a hot air oven. The dried mycelial weight was recorded. The diameter of the plates was measured and the results were analysed statistically.

**Effect of Hydrogen Ion Concentration on the Growth of *Alternaria alternata***

The growth of the pathogen was tested at five different pH levels, *viz*., 4, 5, 6, 7 and 8, respectively. The hydrogen ion (pH) concentration of the Potato Dextrose broth and Potato Dextrose Agar was determined by using a pH meter. pH adjustments were done using 0.1 N alkali (Sodium hydroxide) and 0.1 N acid (Hydrochloric acid). The media was then

sterilised in an autoclave at 121.6 °C for 15 minutes. 100 ml flasks containing 50 ml of sterilised potato dextrose broth were inoculated with a 5 mm disc of the pathogen. Simultaneously, Petri plates containing sterilised 15 ml of potato dextrose agar medium were inoculated with 5 mm discs of the pathogen. The conical flasks and Petri plates labelled with different pHs were then incubated for two weeks and one week, respectively. The experiment was conducted using a Completely Randomized Design (CRD) and each treatment was replicated four times. The mycelial mat in PDB was harvested by filtering through Whatman No.1 filter paper and dried in a hot air oven. The dried mycelial weight was recorded. The diameter of the fungal colony was measured and the results were analysed statistically.

## Results and Discussion

### Sample Collection, Fungal Isolation and Pathogenicity Assay

Aloe vera is one of the most important medicinal plants in Indian gardens with promising medicinal properties. The prevalence of various *Alternaria* species, particularly those responsible for leaf spot diseases, has had a profound and detrimental impact on global crop yields, establishing *Alternaria* as one of the most formidable and destructive pathogens globally. Notably, prior to our investigation, there had been documented instances of *Alternaria alternata* associated with aloe vera in various countries, including India (Tamil Nadu) (Kamalakannan *et al*., 2008), Iran (Abkhoo *et al*., 2014), Pakistan (Rasheed *et al*., 2019) and Bangladesh (Ahmmed *et al*., 2020).

The leaf spot symptoms started as reddish-brown pinpoint lesions with red hallow which later turned into circular to oval dark brown sunken spots with grey margins surrounded by brown boundaries. The mature spots often developed shot holes. The spots mostly appeared on the leaf tips. In the later stage of infection, the diseased leaves exhibited complete drying in an apical-proximal direction (Fig. 1). Kamalakannan *et al*. (2008) noticed similar symptoms on aloe vera leaves collected from Coimbatore, Erode and Madurai districts of Tamil Nadu, India.

a. Field symptoms

b. Symptom progression on the leaves

Fig. 1 : Symptomatology of *Alternaria alternata* leaf spots on aloe vera

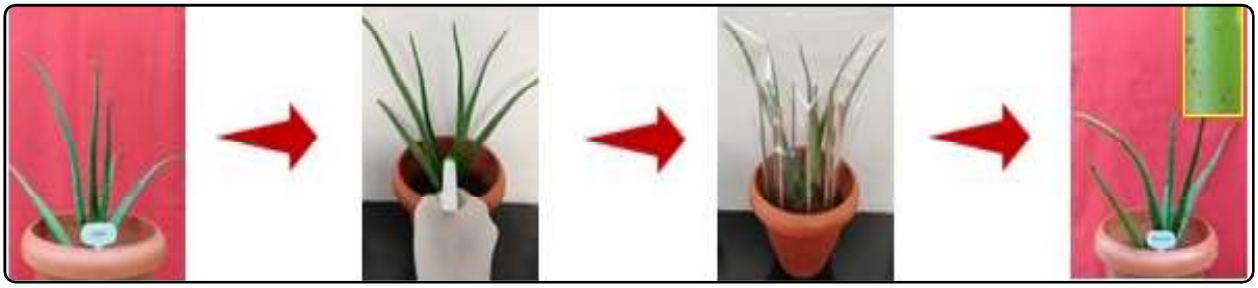
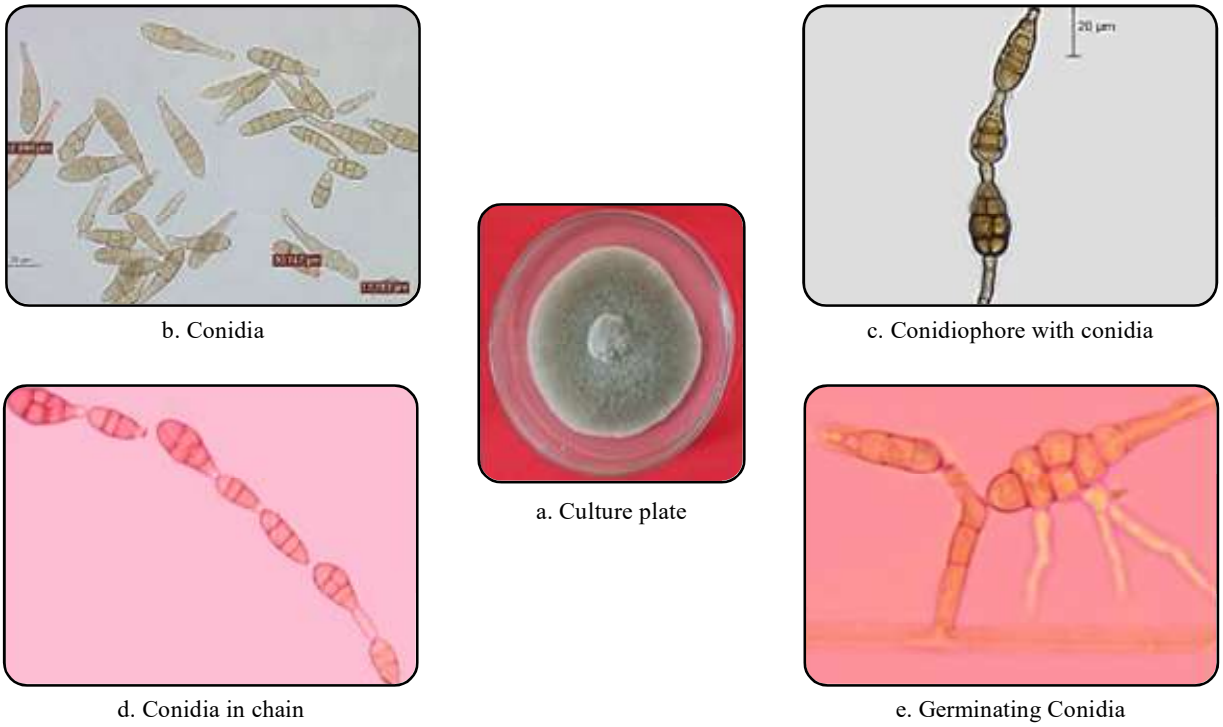


Fig. 2 : Pathogenicity test of *Alternaria alternata*

Most plants within the medicinal garden at GKVK, Bengaluru, India exhibited distinctive leaf spot symptoms with a per cent disease index (PDI) exceeding 50 per cent (n = 50). When pathogenic isolation was performed, *Alternaria*-like colonies were consistently obtained on PDA. The patho genicity test for the fungal isolate was conducted in controlled conditions and Koch's postulates were established. Inoculated leaves exhibited reddish-brown pinpoint lesions with red hallow five days post-inoculation. These spots gradually expanded into circular to oval dark brown sunken spots, while the control plants remained free of any symptoms (Fig. 2). The symptoms induced in the pathogenicity test closely resembled those observed in the field conditions. Re-isolation of the pathogen from infected tissue, coupled with morphological and NCBI-Nucleotide-BLAST analysis, ultimately identified *Alternaria alternata* as the causative agent responsible for leaf spots in aloe vera.

**Morphological Characterization**

Ten-day-old colonies on PDA produced circular umbonate grey aerial mycelium with an entire margin. In reverse, distinct ring patterns have been observed (Fig. 3a). Mycelia were septate. Conidiophores were golden brown**,** single or in small groups, straight or curved, sometimes geniculate,




b. Conidia

a. Culture plate

c. Conidiophore with conidia

d. Conidia in chain

e. Germinating Conidia

Fig. 3 : Micro-morphological characteristics of *Alternaria alternata*

with scars, septate, and ranging from 3-6 µm × 20-70 µm (Fig. 3c). Conidia were golden brown, obclavate, 1-6 transverse and 0-2 longitudinal septate (9-16 µm × 18-62 µm ($n$ = 20)) and often with a short conical or cylindrical beak 2-6 µm in diameter tapering to the apex or blunt, about one third or one-quarter of the conidial length (Fig. 3b, d, and e). These observations closely aligned with the morphological traits outlined in previous studies on *Alternaria alternata* by Kamalakannan *et al*. (2008), offering valuable insights into the genus-level identification of the fungal pathogen as an *Alternaria*.

**Molecular Characterisation**

Further, molecular confirmation was carried out using ITS sequencing and NCBI-BLAST analysis. NCBI-BLASTn of ITS sequences analysis showed homology of 99.82 per cent with *Alternaria alternata*, which solidified the attribution of *Alternaria alternata* as the causative agent of leaf spot in aloe vera within Karnataka, India. Notably, the subtropical climate of India, characterised by high humidity levels, provides an ideal environment for the persistence of pathogens associated with leaf spot diseases.

**Effect of Different Solid Media on the Growth of *Alternaria alternata***

The growth of *Alternaria alternata* was studied on ten different solid media; results were noted in Table 1, Fig. 4 and Plate 1. *Alternaria alternata*

**TABLE 1**

**Effect of different cultural media on the growth of *Alternaria alternata***

| Different media | Radial growth (mm)* | Colony form and elevation | Margin of colony | Zonation | Colony colour | Pigmentation | Sporulation |
|---|---|---|---|---|---|---|---|
| Corn meal agar | 79.67 (63.18) | Circular umbonate | Entire | No zonation | Grey | Yellowish black | +++ |
| Malt extract agar | 50.67 (45.36) | Circular umbonate | Entire | No zonation | Dark grey | Black | + |
| Rose bengal agar | 48.00 (43.84) | Circular umbonate | Entire | No zonation | Dark grey | Black | ++ |
| Czapek's Dox agar | 77.33 (61.55) | Filamentous flat | Entire | Fairly distinct concentric zonation | Whitish grey | Brownish black | +++ |
| Oat meal agar | 87.00 (68.85) | Filamentous flat | Entire | Poorly distinct concentric zonation | Greyish white | Black | +++ |
| Potato Carrot agar | 75.67 (60.43) | Circular umbonate | Entire | No zonation | Grey | Black | +++ |
| Potato dextrose agar | 67.67 (55.32) | Circular umbonate | Entire | No zonation | Grey | Yellowish Black | ++++ |
| Richard's agar | 90.00 (71.55) | Filamentous flat | filiform | Fairly distinct concentric zonation | Greyish brown | Black | ++ |
| Sabouraud dextrose agar | 55.33 (48.04) | Irregular umbonate | Entire | Poorly distinct concentric zonation | Whitish | Greenish black grey | + |
| V8-juice agar | 60.67 (51.14) | Irregular raised | Entire | Poorly distinct concentric zonation | Greyish | Black white | ++++ |
| S.Em ± | 0.43 | | | | | | |
| CD @ 1% | 1.72 | | | | | | |

* Figures in parenthesis indicate the mean of arc-transformed values

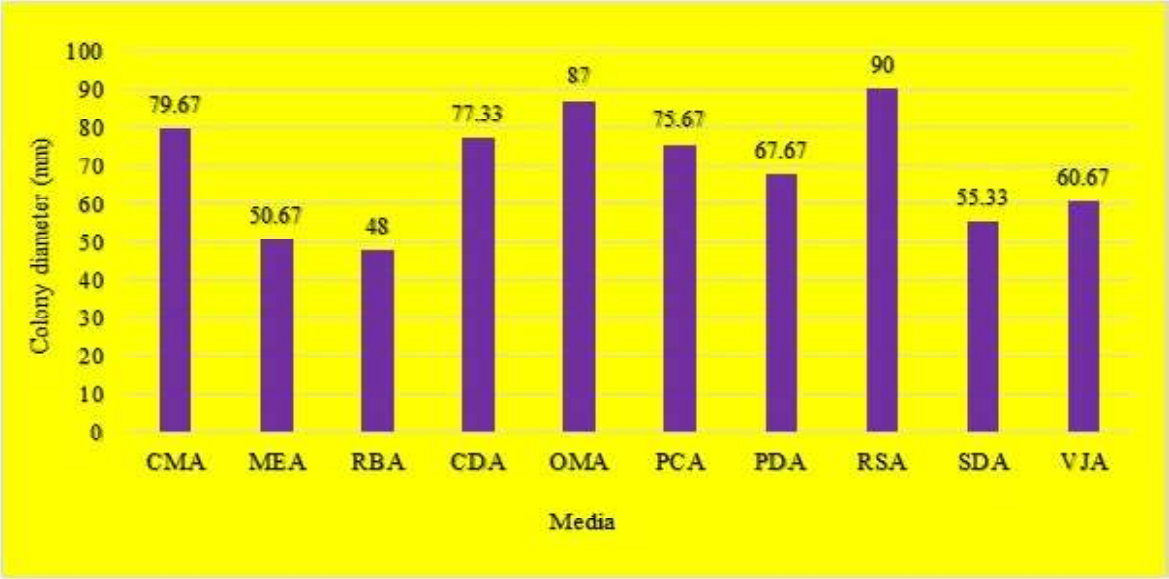



Fig. 4 : Growth of *Alternaria alternata* on different cultural media

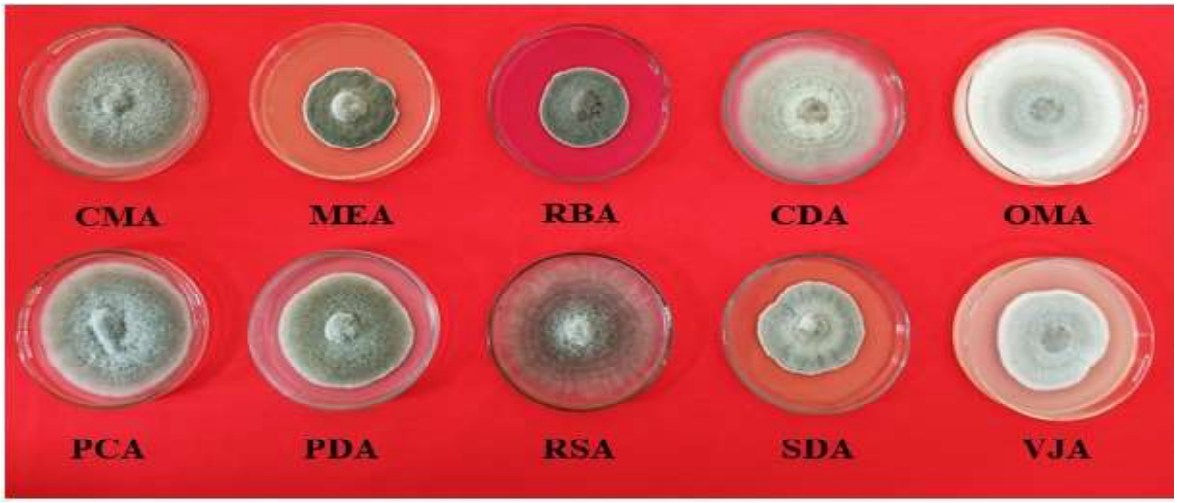



Plate 1 : Growth of *Alternaria alternata* on different cultural media

growth varied among different media tested and it ranged from 48.00 mm to 90.00 mm. The maximum radial growth of *Alternaria alternata* was recorded on Richard's Synthetic Agar (90 mm) which was followed by Oat Meal Agar (87 mm) and Corn Meal Agar (82.81 mm). The minimum radial growth of *Alternaria alternata* was recorded on Rose Bengal agar (48.00 mm), followed by Malt Extract Agar and Sabouraud Dextrose Agar. Similarly, Choudhary *et al*. (2017) reported Richard's Synthetic Agar as one of the best mediums and Rose Bengal Agar as the worst medium for the growth of *Alternaria alternata* causing leaf blight of Isabgol. Ahmmed *et al*. (2020) recorded the highest mean colony diameter of *Alternaria alternata* on Potato Dextrose Agar, Potato Carrot agar and Richard's Synthetic Agar, respectively. The cultural characteristics of *Alternaria alternata*, such as colony diameter, colony form and elevation, colony margin, zonation, colony colour, pigmentation and sporulation varied in each medium. *A. porri* grown on different media, such as V8, PDA, OMA and MEA, showed the highest spore production in the V8 medium, followed by the PDA, OMA and MEA medium (Kim *et al*., 2022). These findings agreed with our results.

**Physiological Studies**

**Effect of Temperature on Growth of *Alternaria alternata***

The experiment was done to know the optimum temperature for the growth of *Alternaria alternata.* In this study, different temperature levels *viz.,* 15°C, 20°C, 25°C, 30°C and 35°C were tested. The results are presented in Table 2, Fig. 5-6 and Plate 2-3. *Alternaria alternata*'s growth in PDB gradually increased from 15 to 30 °C and later, it decreased at increasing temperatures. The growth differences observed at all temperatures were statistically significant with each other. Maximum mycelial dry weight was obtained at 30°C (161.53 mg), followed by 25°C (143.37 mg), and minimum mycelial dry weight was obtained at 15°C (54.2 mg), followed by at 20°C (112.57 mg).

**TABLE 2**

**Effect of temperature on the growth of *Alternaria alternata***

| Temperature | Potato dextrose broth | Potato dextrose agar | | |
|---|---|---|---|---|
| ($^0$C) | Dry mycelial weight (mg)* | Radial growth (mm)* | Mycelial colour | Type of growth |
| 15 | 54.2 | 22.33 (28.19)* | Greyish white | Circular raised |
| 20 | 112.57 | 45.00 (42.11)* | Greyish white | Circular raised |
| 25 | 143.37 | 55.67 (48.23)* | Greyish white | Circular raised |
| 30 | 161.53 | 58.67 (49.97)* | Greyish white | Circular raised |
| 35 | 33.30 | 13.33 (21.35)* | Greyish white | Circular raised |
| S.Em ± | 0.70 | 0.59 | | |
| CD @ 1% | 3.12 | 2.63 | | |

* Figures in parenthesis indicate the mean of arc-transformed values

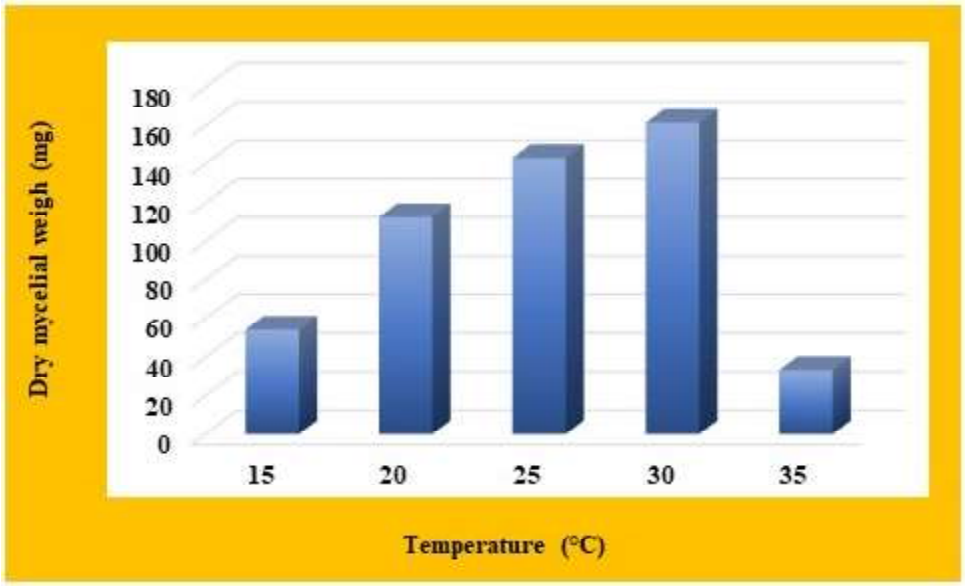



Fig. 5 : Effect of temperature on the growth of *A. alternata* on PDB

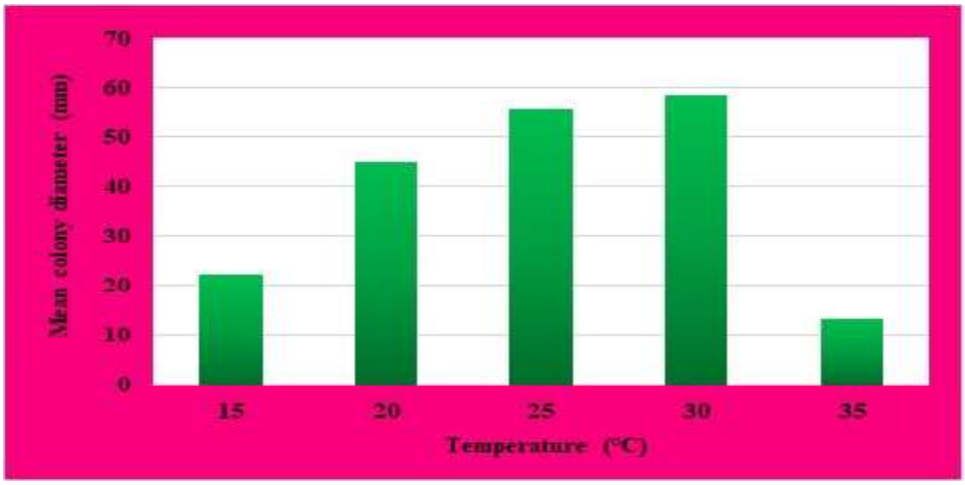



Fig. 6 : Effect of temperature on the growth of *A. alternata* on PDA

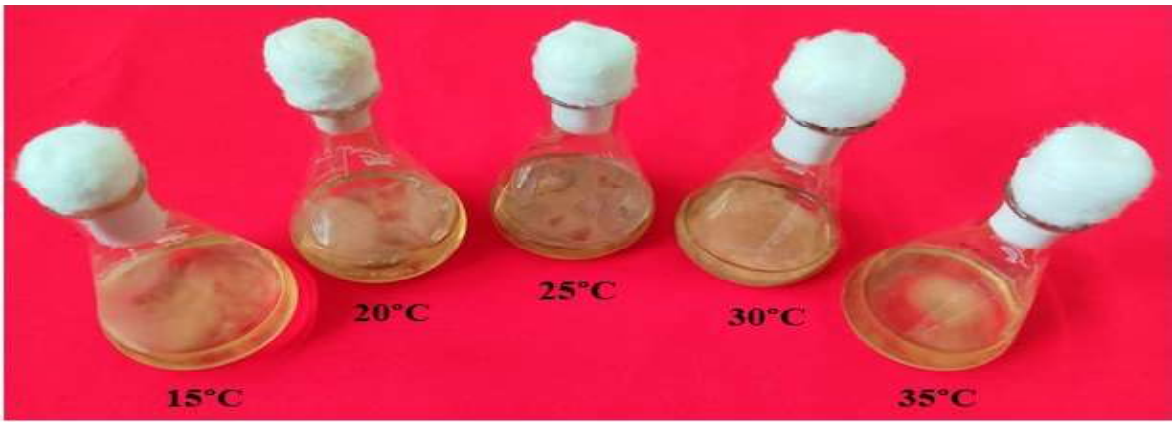



Plate 2 : Effect of temperature on the growth of *A. alternata* on PDB

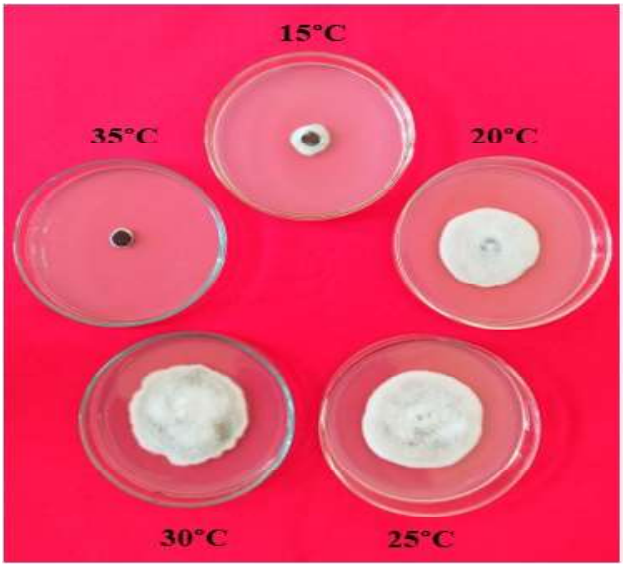



Plate 3 : Effect of temperature on the growth of *A. alternata* on PDA

A similar pattern of growth of *Alternaria alternata* was observed in PDA plates. Abkhoo *et al*. (2014) evaluated the effect of temperature on the mycelial growth of 15 isolates of *Alternaria alternata* associated with leaf spots in aloe vera. All 15 isolates grew well at 25°C (58.2 mm), followed by 30°C (57.1 mm). The most negligible growth was observed at 5°C (5.5 mm). From the study, it can be concluded that temperature ranging from 25 to 30°C is better for the growth of *A. alternata*. The results are supported by Marín *et al*. (2006) and Garibaldi *et al*. (2007), who reported that 27°C was the optimum temperature for the growth of *A. alternata.* In addition, Tu *et al*. (2016) and Ngoc *et al.* (2013) also observed the 30°C as an optimum temperature for *Alternaria* sp., which solidified the result.

**Effect of pH on the Growth of *Alternaria alternata***

The study was conducted to know the optimum pH level required for the growth of *Alternaria alternata*. Observations were taken at different pH levels, *viz.,* 4.0, 5.0, 6.0, 7.0 and 8.0. Results are shown in Table 3, Fig. 7-8 and Plate 4-5. The highest *Alternaria alternata* dry mycelial weight was recorded at the pH of 7.0 (182.90 mg), followed by pH 6.0 (162.50 mg) and the growth was seen least at 4.0 pH (83.03 mg) and 5.0 (120.93 mg). The radial mycelial growth of *Alternaria alternata* on potato dextrose agar plates at different pH levels also exhibited a similar trend. Previous studies conducted by Hubballi *et al.* (2010), Azad *et al*. (2016) and Nira *et al*. (2021) revealed that the best temperature and pH for the growth of *A. alternata* and other *Alternaria* spp. is 25-30 °C and 6-7 pH, respectively, supporting this study's findings.

**TABLE 3**

**Effect of hydrogen ion concentration (pH) on growth of *Alternaria alternata***

| pH level | Potato dextrose broth | Potato dextrose agar | | |
|---|---|---|---|---|
| | Dry mycelial weight (mg)* | Radial growth (mm)* | Mycelial colour | Type of growth |
| 4 | 83.03 | 28.33 (32.14)* | Greyish white | Circular raised |
| 5 | 120.93 | 45.67 (42.50)* | Greyish white | Circular raised |
| 6 | 162.50 | 62.00 (51.97)* | Greyish white | Circular raised |
| 7 | 182.90 | 71.67 (57.83)* | Greyish white | Circular raised |
| 8 | 141.77 | 56.00 (48.43)* | Greyish white | Circular raised |
| S.Em ± | 0.86 | 0.55 | | |
| CD @ 1% | 3.84 | 2.45 | | |

* Figures in parenthesis indicate the mean of arc-transformed values

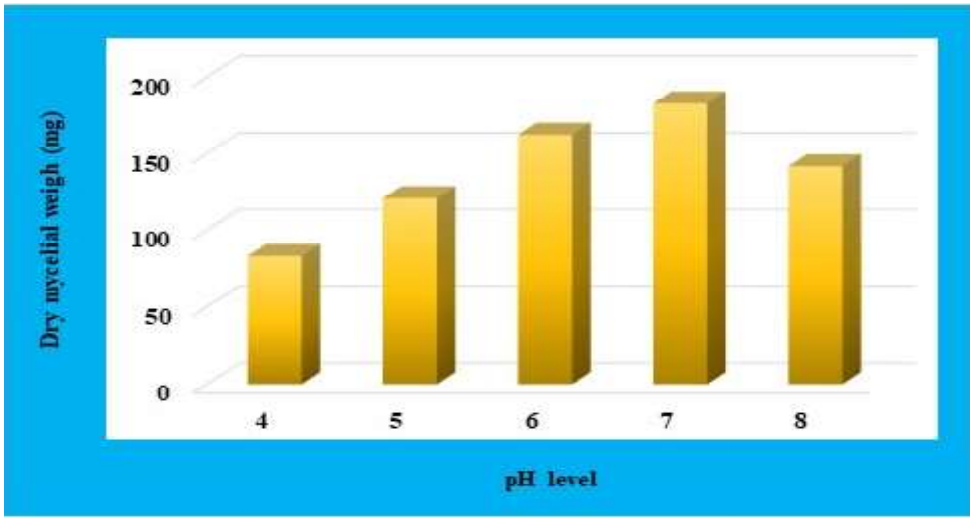



Fig. 7 : Effect of pH on the growth of *A. alternata* on PDB

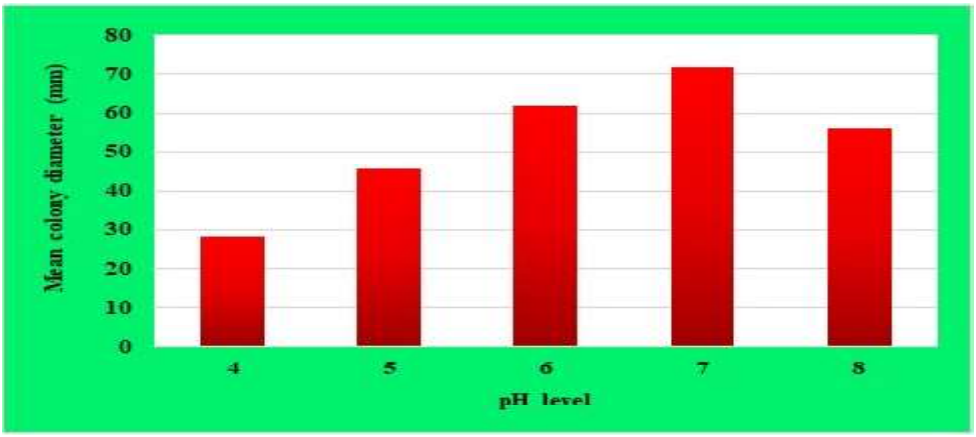



Fig. 8 : Effect of pH on growth of *A. alternata* on PDA

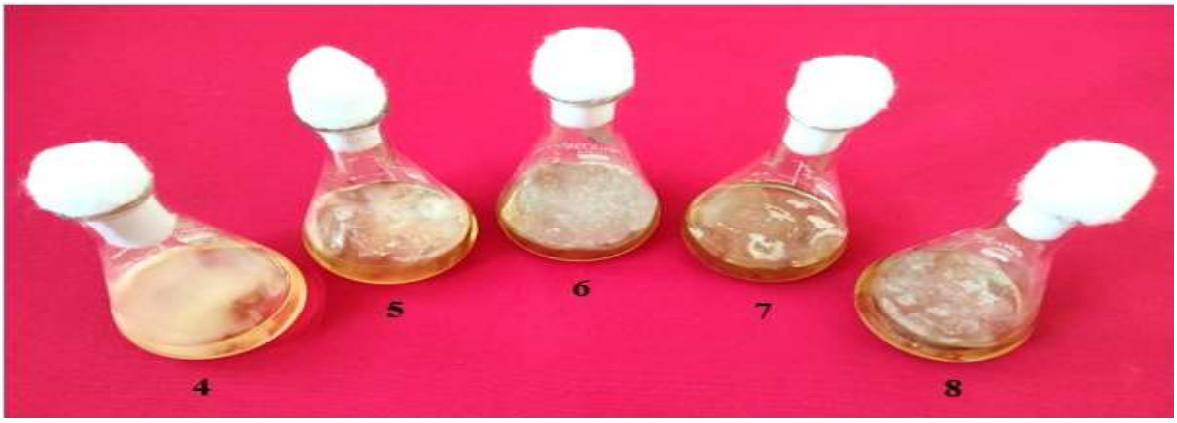



Plate 4 : Effect of pH on the growth of *A. alternata* on PDB


Plate 5 : Effect of pH on growth of *A. alternata* on PDA

This study comprehensively elucidates Alternaria leaf spot disease of aloe vera in Karnataka, India. Through morphological, molecular and physiological characterisations, the study confirms the identity of *Alternaria alternata* and provides valuable insights into pathogen identification and disease epidemiology. The research findings contribute to advancing knowledge in plant pathology and lay the foundation for better managing Alternaria leaf spot disease in aloe vera. Future research directions may focus on exploring management measures and developing resistant cultivars to mitigate the impact of this devastating disease.

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