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Assessment of different culture media on the growth and sporulation of *Alternaria alternata* causing leaf spot of bael (*Aegle marmelos* Correa)

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Abstract
The primary disease affecting bael plants is *Alternaria* leaf spot, which is caused by *Alternaria alternata.* This disease is increasingly posing a significant challenge in Uttar Pradesh and other regions of the country. In laboratory conditions, the fungus demonstrates robust growth in potato dextrose agar and Bael leaf extract agar mediums. To compare the effects of different solid media on fungal growth, researchers conducted experiments using eight variations. The findings revealed that Potato Dextrose Agar medium exhibited the most substantial mycelium growth, measuring 88.88 mm. Following closely was Beal leaf extract agar medium with 72.00 mm of growth, while Czapeck's Dox agar and Richard's agar mediums showed similar effectiveness with growth measurements of 69.13 mm and 67.60 mm, respectively. Conversely, Martins media displayed the least favorable outcome, supporting a mere 40.15 mm of mycelium growth. These findings highlight that the radial growth of *A. alternata* depends on the culture media used, with different media influencing the growth rate and pattern of the fungus, resulting in variations in radial growth. The data suggest that, in general, the growth rate increases with time for all media tested, but some media promote faster growth than others. Moreover, sporulation was found to be most favorable in potato dextrose agar and Bael leaf extract agar mediums. Consequently, it can be concluded that potato dextrose agar is the most suitable medium for the growth and sporulation of *Alternaria alternata*, which causes leaf spot on bael plants (*Aegle marmelos* Correa).

Keywords: *Alternaria alternata*, *Aegle marmelos* correa, different culture media, growth behavior, bael

Introduction
In India, *Aegle marmelos*, also referred to as the "Bael tree," is a prominent and respected plant. It is highly valued in the nation's cultural, religious, and medical traditions. The Indian fruit known as bael (*Aegle marmelos* Correa) is a member of the Rutaceae family and has the chromosomal number 2n=36 (n=18). It is also known by the names Bengal queen, Golden apple, Japanese sour orange, Stone apple, and wooden apple (John and Stevenson, 1979) [8]. However, this deciduous tree is indigenous to the Indian subcontinent and is found in many different Indian states. Bael also grown and well proliferated in the wild and semi-wild in Uttar Pradesh, Rajasthan, Gujarat, Bihar, West Bengal and Madhya Pradesh (Singh and Choudhary, 2012) [13]. In Uttar Pradesh usually in Mirzapur, Varanasi, Gorakhpur, Basti, Gonda, Ayodhya, Etawah districts, and additionally Sewan district of Bihar (Teaotia *et al*., 1963) [14]. Though bael plants are hardy and tolerant to biotic and abiotic stresses in the ancient era but in the modern period these are also attacked by microbial pathogens and causing severe menace. The important diseases of bael plants are as follows. Alternaria leaf spot caused by *Alternaria alternata* (Madaan and Gupta, 1985) [10], Myrothecium leaf spot caused by *Myrothecium roridum* (Harsh *et al*. 1989) [6], Bacterial shot hole and fruit canker caused by *Xanthomonas bilvae* (Patel *et al*. 1953) [15], Stalk end rot caused by *Fusarium solani* (Bhargava *et al*. 1977) [2], Aspergillus fruit rot caused by *Aspergillus awamori* (Arya *et al*. 1986) [1], Fusarium fruit rot caused by *Fusarium moniliformae* (Arya *et al*. 1986) [1], Powdery mildew caused by *Oidium* sp. and *Sphaerotheca fuliginea* (Sinha and Singh,1995) [12], Scytalidium leaf spot (brown leaf spot) caused by *Scytalidium aeglicola* (Gautam *et al*. 2015) [4] Among these, Alternaria leaf spot of bael is now becoming an important menace in Uttar Pradesh and other parts of the country. Madaan and Gupta (1985) [10] first time reported leaf spot disease of bael, caused by *Alternaria alternata* (Fr.) Keissler from Hisar, Haryana.

Only a small amount of information is provided in India, particularly in the state of Uttar Pradesh, according to an analysis of works done on bael diseases. Therefore, the following goals can be pursued while keeping in mind the relevance of the bael crop and the impact of leaf spot on bael diseases caused due to *Alternaria alternata*. Hence, present investigations were undertaken to check the best suitable media for the growth of *Alternaria alternata* causing leaf spot of bael (*Aegle marmelos* Correa). *Alternaria alternata*, despite being a widespread fungus, necessitates specific compounds for its growth. To investigate its growth, nutrition, physiology, and management, researchers isolate the fungus as a pure culture in particular media during *in vitro* studies. A diverse array of media can facilitate the isolation of *A. alternata*, supporting its radial growth, dry weight growth, and sporulation. However, the nutritional needs for promoting fungal growth may not align with those required for optimal sporulation. Moreover, different media compositions can influence the colony morphology of *A. alternata*, which is essential for morphological characterization, a classical approach used in distinguishing fungal species and an integral aspect of fungal taxonomy.

Material and Method

Isolation of pathogen

Diseased samples were taken from infected Beal plant from the Main Experiment Station, Department of Horticulture and Department of Plant Pathology, Acharya Narendra Deva University of Agriculture & Technology, Kumarganj, Ayodhya, Uttar Pradesh (26.5412º N, 81.8320º E). Diseased samples with distinct disease characteristics were intentionally selected to isolate the pathogen. The chosen plant parts underwent a thorough washing process using freshly sterilized water to remove any surface contaminants or dust particles. Careful precision was exercised while cutting the washed diseased plant parts into small pieces, ensuring that some healthy portions were included. These cut pieces were then subjected to surface sterilization within aseptic conditions, using a 1.0% Sodium hypochlorite solution, within a Laminar flow hood. Afterward, they underwent multiple cycles of rigorous washing with sterilized water to eliminate any remaining traces of Sodium hypochlorite. Excess moisture was eliminated by placing the sterilized pieces on sterilized blotting papers. Once sterilized, the pieces were transferred to Petri dishes using sterilized needles. The Petri dishes had been previously sterilized at 180°C for twenty minutes in an electric hot air oven and were filled with a 2% potato dextrose agar medium. The medium itself had been autoclaved at a pressure of 15 pounds per square inch for 30 minutes. Within each Petri dish, three to four pieces of the diseased plant parts were positioned at equal distances from one another. Proper labelling was done on the Petri dishes using a gloss marking pencil to indicate the date of isolation, isolate number, and other relevant details. Finally, the Petri dishes were transferred to an incubator set at a temperature of 25±1ºC.

Pure culture of the pathogen

The fungus was subjected to purification using the single spore isolation technique. A diluted spore suspension was evenly spread on plain agar in Petri dishes, creating a thin layer that allowed the spores to settle on the agar surface. Once settled, individual spores were carefully isolated from one another, examined under a microscope, and marked using a dummy cutter within the Petri dishes. The marked spores, along with agar blocks, were then transferred to Petri dishes containing sterilized 2% potato dextrose agar medium. After the fungi obtained from the single spore culture exhibited satisfactory growth, regular sub-culturing was carried out to monitor any potential contamination until a pure culture was obtained. These cultures underwent sub-culturing every 15 days and were maintained on potato dextrose agar slants under refrigeration at temperatures ranging between 6 to 8 °C for further research purposes.

Maintenance and preservation of culture

To maintain various cultures of *Alternaria alternata*, sterilized Potato Dextrose Agar (PDA) was utilized as the growth medium. Petri dishes were inoculated with a small portion taken from 10-day-old cultures that had been grown on PDA at a temperature of 25±1 ºC. Following an incubation period of 20 days, one set of each pathogen was regularly sub-cultured on PDA. Additionally, a separate set of pure cultures was stored in the refrigerator to preserve their virulence, which could be compromised by frequent sub-culturing. To encourage fungal sporulation, the cultures were placed in an incubator set at a temperature of 25±1 ºC.

Effect of different solid media on mycelial growth

To assess the growth of the fungus on solid media, the researchers measured the colony's diameter, excluding the initial 5 mm diameter of the disc, along with two diagonals passing through the colony's center. For their *in vitro* investigations, they employed eight different solid media, each with its unique composition (Table 1). The experimental setup involved placing sterilized medium in Petri plates and inoculating them with a 5 mm disc of mycelial growth using a sterilized cork borer. These plates were then placed in an incubator at a constant temperature of 25±1 ºC for a duration of 8 days, and the researchers conducted four replications. After the 8-day incubation period, they observed and recorded the radial growth of the mycelium. The primary objective was to determine which solid media were most suitable for promoting the growth of the test fungus. All assessments were carried out at a consistent temperature of 25±1 ºC using the eight different solid media.

Table 1: Constituents of different media

S. No.	Medium	Constituents	Quantity
1.	Potato Dextrose Agar	Agar agar	20.00 g
		Dextrose	20.00 g
		Peeled potato	250.00 g
		Distilled water	1000 ml
2.	Czapeck's Dox Agar medium	Agar agar	15.00 g
		Sucrose	30.00 g
		Distilled water	1000 ml
		Dipotassium	1.00 g
		phosphate Magnesium	0.50 g
		Potassium chloride	0.50 g
		Sodium nitrate	2.00 g
		Ferrus sulphate	0.01 g
3.	Oat meal agar medium	Agar agar	15.00 g
		Rolled oats	30.00 g
		Distilled water	1000 ml
4.	Corn meafl agar medium	Agar agar	20.00 g
		Corn meal	20.00 g
		Glucose	20.00 g
		Distilled water	1000 ml
5.	Martin's medium	Agar - agar	15.00 g
		Dextrose	10.00 g
		Peptone	5.00 g
		Distilled water	1000 ml
		Rose Bengal	1 part in 3000 parts of the media
		Magnesium sulphate	0.50 g
		Potassium dihydrogen phosphate	1.00 g
6.	Richard's Agar	Potassium nitrate	10.00 g
		Potassium monobasic phosphate	5.00 g
		Magnesium sulphate	2.50 g
		Ferric chloride	0.02 g
		Sucrose	50.00 g
		Agar	20.00 g
7.	Waksman Agar	Potassium monobasic phosphate	1.00 g
		Magnesium sulphate	0.50 g
		Glucose	10.00 g
		Bacto-peptone	5.00 g
		Agar	20.00 g
8.	Bael leaf extract medium	Agar agar	20.00 g
		Dextrose	20.00 g
		Beal leaf extract	200.00 g
		Distilled water	1000 ml

Colony and growth characters

The appearance of the colony, the color of both the colony and substrate, the colony's margin, and the mycelium's topography were all observed using the naked eye. To measure sporulation on various media, a single 5 mm diameter block was carefully excised from the fungal colony near the margin using a sterilized cork borer. This block was then transferred to a test tube containing 5 ml of sterile distilled water, where it was thoroughly mixed to create a uniform spore suspension. A small drop of the spore suspension was placed on a microscope slide, and the average spore count was recorded by observing three microscopic fields under the low-power (10X) objective of the microscope. The cultural and morphological characters of the fungus were recorded on a PDA medium after 2 to 8 days of incubation. Colour, Growing pattern and type of mycelium were observed with the help of a binocular light microscope.

Statistical Analysis

The experiments were conducted in a controlled laboratory environment, ensuring consistent and regulated conditions. The data obtained from the experiments were analyzed using a statistical approach known as the completely randomized design (CRD). This design allows for random allocation of treatments or variables, reducing potential biases and increasing the validity of the analysis.

Result and Discussion

Effect of different culture media on colony and growth characters of *Alternaria alternata* infecting bael.

Radial growth of *Alternaria alternata* on different solid culture media

Some specific elements and compounds are required for the growth of the fungus, so it is important to provide those essential compounds in the medium to proliferate the fungi in the laboratory. In this above experiment, eight different solid media were evaluated for the growth of *Alternaria alternata*. On perusal of data (Table 2 and Fig 1) revealed that Potato Dextrose Agar medium was found most supportive for the mycelium growth of the pathogen which showed maximum (88.88 mm) growth of the mycelium followed by Beal leaf extract agar medium (72.00 mm) followed by Czapeck's Dox

agar medium (69.13 mm) and Richard's agar medium (67.60 mm) where at par with each other. While the least favourable was Martins media (40.15mm). These findings indicate that the radial growth of *A. alternata* varies depending on the culture media used. Different media can influence the growth rate and pattern of the fungus, leading to variations in its radial growth. The data suggests that the growth rate generally increases with time for all media tested, with some media promoting faster growth than others. Similarly, the result found by Hubballi *et al*. (2010) [7] recorded better growth of *A. alternata* on different media and among them, the host leaf extract medium supported maximum growth followed by potato dextrose agar (PDA). Krishna *et al.* (2018) [9] conducted an experiment on 11 different solid culture media with *A. dauci* causing blight of carrot and recorded maximum mycelial growth on PDA (90.00 mm), followed by potato carrot agar (75.67 mm), oat meal agar (70.67 mm), carrot root agar (62.41 mm), carrot leaf extract agar (54.00 mm), Richard's agar (51.67 mm) and Czapeck's Dox agar (50.67 mm). Choudhary *et al*. (2020) [3] conducted an experiment on some physiological parameters including growth media and recorded the best growth of *Alternaria alternata*, causing Alternaria leaf spot of lehsua (*Cordia myxa*) on potato dextrose agar medium (87.88 mm dia. colony) followed by Czapeck's Dox agar medium.

Table 2: Radial growth of *Alternaria alternata* on different solid culture media

Name of culture media	Mycelia growth after Inoculation in mm			
	48 hr	96 hr	144 hr	192 hr
Corn meal agar medium	15.40	32.20	42.50	55.90
Czapeck's Dox agar medium	19.50	37.60	54.20	69.13
Oat meal agar medium	17.20	36.90	52	61.00
Martin's agar medium	10.90	23.2	34.12	40.15
Potato dextrose agar medium	38.60	59.60	75.20	88.88
Richard's Agar medium	17.00	36.40	48.00	67.60
Waksman Agar medium	16.20	34.00	46.30	65.00
Bael leaf extract agar medium	22.20	40.30	56.00	72.00
SE m±	0.29	0.50	0.69	0.79
CD at 1%	1.15	1.97	2.73	3.14

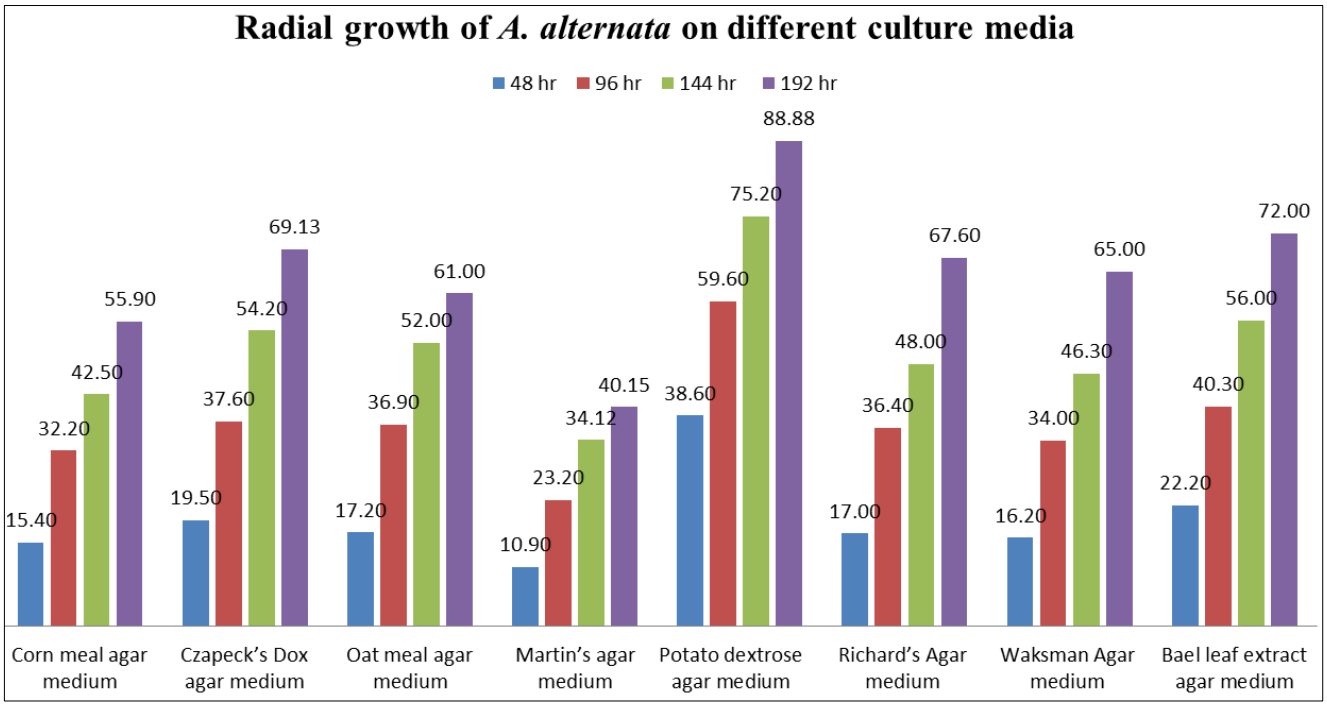

Fig 1: Radial growth of *Alternaria alternata* on different solid culture media

Colony character of Alternaria *alternata* on different solid culture media

In this above experiment, eight different solid media were evaluated for the growth of *Alternaria alternata*. On perusal of data (Table 3 and Plate 1) reveled that Potato dextrose agar medium showed a significantly larger mean mycelial diameter of 88.88 mm. The colony appeared grey, fluffy and exhibited very fast growth with a smooth margin. The sporulation was most abundant indicated by a quadruple plus sign (+ + + +), followed by Bael leaf extract agar medium exhibited a mean mycelial diameter of 72.00 mm. The colony appeared greenish-grey and compressed, with fast growth and a smooth margin. The sporulation was abundant, indicated by a quadruple plus sign (+ + + +), followed by Czapeck's Dox agar medium having a mycelial diameter of 69.13 mm. The colony had a greenish-grey color, appeared fluffy, and exhibited fast growth with a wavy margin. The sporulation was very good indicated by a triple plus sign (+ + +), followed by Richard's Agar medium mycelial diameter of 67.60 mm. The colony appeared whitish-grey and fluffy, with

fast growth and a smooth margin. The sporulation was observed with a moderate amount (+ +), followed by a Waksman Agar medium mycelial diameter of 65.00 mm. The colony appeared dark green and compressed, with fast growth and a smooth margin. The sporulation was observed with a moderate amount (+ +), followed by Oat meal agar medium with a mycelial diameter of 61 mm. The colony appeared dark brown and compressed, with slow growth and a smooth margin. The sporulation was observed with a moderate amount (+ +). While slowest growth and least sporulation were observed in Martin's agar medium mycelial diameter of 40.15 mm. The colony had a whitish-green color, appeared fluffy, and exhibited slow growth with a smooth margin. The sporulation was observed to be fair, indicated by a single plus sign (+), followed by a Corn meal agar medium mycelial diameter of 55.9 mm. The colony appeared green and compressed, with slow growth and a wavy margin. The sporulation was observed with a moderate amount, indicated by a double plus sign (+ +). Similarly, the result was found in Hubballi *et al.* (2010) [7], Krishna *et al.* (2018) [9], Choudhary *et al.* (2020) [3], and Goswami and Mishra, (2022) [5].

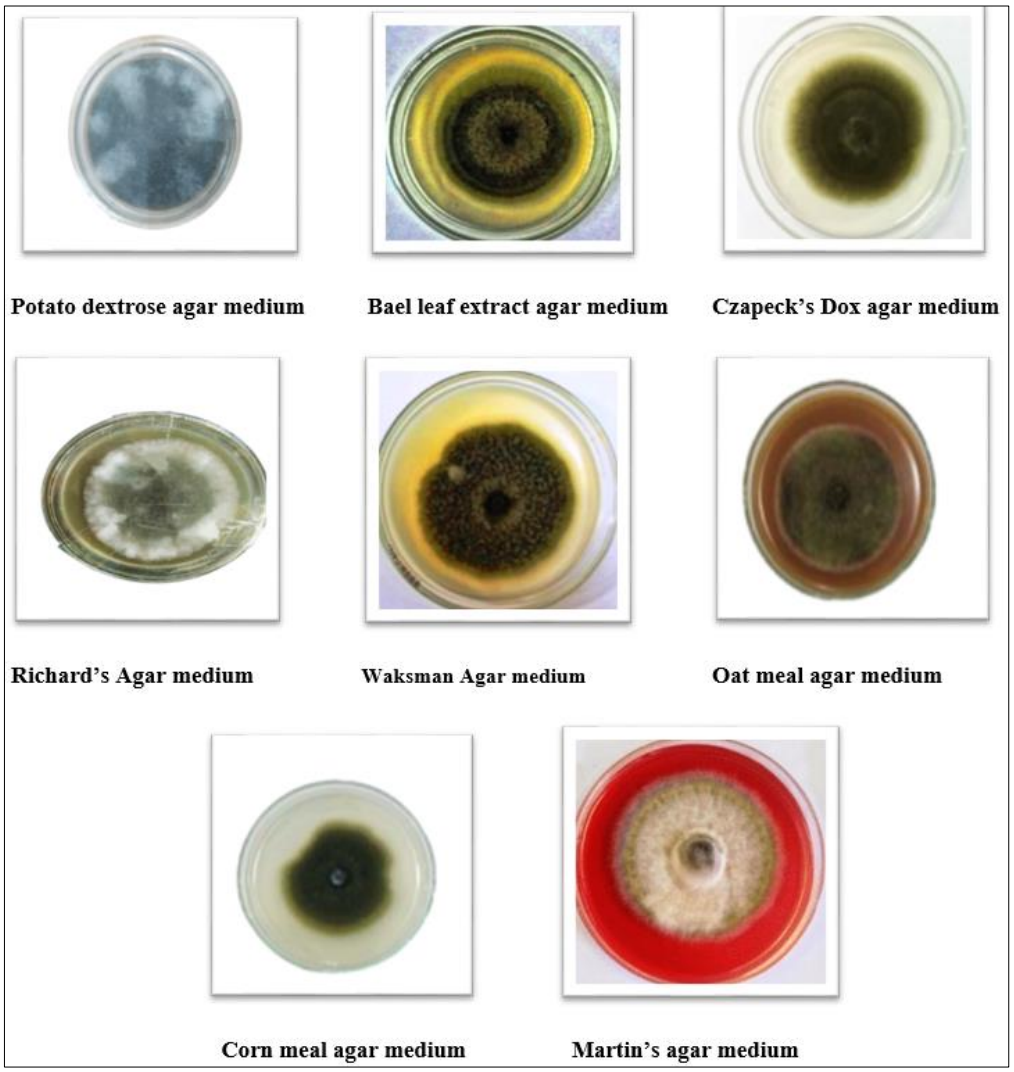

Plate 1: Radial growth of *Alternaria alternata* solid after 192 hr. of inoculation on Different culture media

Table 3: Morphological characteristics of *A. alternata* on different solid media

Sr. No.	Media	Mycelial diameter (mm)	Colony characters				Sporulation
			Color	Appearance	Growth	Margin of colony	
1.	Corn meal agar medium	55.90	Green	Compressed	Slow	Wavy	+ +
2.	Czapeck's Dox agar medium	69.13	Greenish grey	Fluffy	Fast	Wavy	+ + +
3.	Oat meal agar medium	61.00	Dark brown	Compressed	Slow	Smooth	+ +
4.	Martin's agar medium	40.15	Whitish green	Fluffy	Slow	Smooth	+
5.	Potato dextrose agar medium	88.88	Grey	Fluffy	Very fast	Smooth	+ + + +
6.	Richard's Agar medium	67.60	Whitish Grey	Fluffy	Fast	Smooth	+ + +
7.	Waksman Agar medium	65.00	Dark green	Compressed	Fast	Smooth	+ + +
8.	Bael leaf extract agar medium	72.00	Greenish grey	Compressed	Fast	Smooth	+ + + +

+ Fair, 0-10 conidia per microscopic field; + + + Very Good, 20-30 conidia per microscopic field;
+ + Good, 10-20 conidia per microscopic field; + + + + Excellent, more than 30 conidia per microscopic field

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