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CULTURAL CHARACTERISTICS OF PHYLLOPLANE MYCOFLORA AND LEAF SPOT PATHOGEN ASSOCIATED WITH *SOLANUM LYCOPERSICUM* L.

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ABSTRACT

A comprehensive study was conducted to explore the diversity and growth behaviour of phylloplane fungi associated with healthy and diseased tomato (*Solanum lycopersicum* L.) leaves. From the surface of healthy leaves, three fungal species were isolated and identified as *Fusarium* sp., *Aspergillus niger,* and *Aspergillus flavus.* In contrast, symptomatic tomato leaves yielded one pathogenic fungi, namely *Alternaria solani.*. All four fungal isolates were cultured on seven distinct nutrient media: Potato Dextrose Agar (PDA), Czapek's Dox Agar, Oat Meal Agar, Rose Bengal Agar, Richard's Synthetic Agar, Sabouraud's Dextrose Agar, and Malt Extract Agar. The cultures were incubated at 25°C for seven days, following which parameters like radial mycelial growth, colony morphology, and sporulation intensity were carefully assessed. The growth reactions of these phylloplane fungi were analyzed on seven different solid culture media. *Fusarium* sp., *Aspergillus niger,* and *Alternaria solani* exhibited the greatest radial expansion on both PDA and Richard's synthetic agar. Meanwhile, *Aspergillus flavus* demonstrated the highest growth on PDA and Sabouraud's dextrose agar.

Key words : Tomato, Phylloplane fungi, Cultural characteristics, Leaf spot pathogens, Growth media.

Introduction

The tomato, or *Lycopersicon esculentum* Mill., is one of the most prominent and commonly grown vegetable crops in the world, recognized for both its economic and nutritional advantages. It is a significant producer of vegetables in India, but several kinds of diseases, particularly fungal pathogens like *Alternaria solani* (Ellis and Martin), which results in early blight and might result in significant yield losses, severely restricting its productivity (Shamurailatpam *et al*., 1986). The phylloplane mycoflora is an eclectic mix of microorganisms found in the aerial regions of the tomato plant, mainly the leaves. Last (1955) and Ruinen (1956) extended the idea of the phylloplane as a dynamic microbial home by emphasizing the complicated biology of the leaf-surface microflora. Lindow and Brandl (2003) highlighted the ecological role played by phylloplane fungus in managing pathogen acquisition and disease development, whereas (Tsuneda *et al*., 1978) further classified them into active and transient kinds. According to a number of research, such as Cook (1980) and Blakeman and Fokkema (1982), some phylloplane fungi have a capacity to limit foliar pathogens through utilizing competition, antibiosis and other antagonistic mechanisms, indicating that they may be associated with biological management.

For accurate investigation and description of phylloplane mycoflora, isolation and cultivation on suitable solid media are necessary as the amount of nutrients greatly impacts the development of fungus colony morphology, sporulation and pigmentation. Cultural characteristics seen on artificial media serve as crucial

for fungal identification, according to Barnett and Hunter (1972). According to Booth (1971) and Griffin (1994) various solid media, including Potato Dextrose Agar, Czapek's Dox Agar and Malt Extract Agar, offer numerous sources of carbon and nitrogen, which impacts the physiological variability and growth behavior of fungal isolates. Considering the development of phylloplane mycoflora using different solid media additionally makes precise identification easier, assist in finding of promising isolates to conduct further pathogenic and biocontrol research. The present investigation was carried out to analyze the growth of tomato phylloplane fungus in different solid media for the purpose to develop greater knowledge of their physiological characteristics as well as potential uses in disease control, given the lack of information at present on their cultural behavior.

Materials and Methods

Collection, isolation and purification of phylloplane mycoflora from healthy leaves

Healthy leaf samples were collected from multiple tomato plants at the college farm of OUAT. From each plant, healthy leaves were carefully chosen and collected for the purpose of isolating pathogens. These leaf samples were then stored in labelled paper bags until the microorganism isolation process was conducted. Leaf washing method (Buri, 1903) was followed for isolation of mycoflora from phylloplane of tomato. Forty small leaf segments, each measuring 5.0×5.0 mm, were skilfully sliced using a heated razor blade from the lower leaves of the tomato plant. These leaf pieces were carefully placed into a sterilized conical flask of 250 ml capacity, which already contained 100 ml of pure and sterile distilled water. To ensure comprehensive cleaning of any fungal particles on the leaf surfaces, the flask was vigorously shaken for a duration of 20 minutes.

Subsequently, one mm of the resulting leaf washings was carefully moved into a sanitized Petri-dish filled with Martin's agar medium. This process was repeated across five Petri-dishes, each serving as a replication. The Petri-dishes, now containing the inoculated culture plates, were then left to incubate at room temperature (approximately 28-21 degrees Celsius). After a 4-day incubation period, the number of colonies belonging to various types of fungal species that had thrived on the agar medium were meticulously counted using a colony counter. The diverse fungal isolates were then meticulously transferred to agar slants using aseptic techniques for further examination and analysis. After incubation, the plates were examined for the presence of microbial growth. Any visibly distinct fungal colonies with unique morphological characteristics from each plate were isolated and transferred to fresh PDA plates. These isolates were then purified through repeated subculturing. The pure cultures were identified using their scientific names and subsequently transferred to culture slants containing PDA for storage at a temperature of 4°C.

Collection, isolation and purification of pathogen from infected leaves

Naturally infected leaves were collected from the farmer's field and from horticulture department OUAT, Bhubaneswar. A thorough washing with distilled water was performed on the leaves that were brought into the lab. In order to preserve the healthy and diseased portions, leaves were blot dried before being chopped into small (5mm) pieces using a sharp, sterilised blade. The surface of the diseased sample was sterilised for two seconds with 0.1 percent mercuric chloride ($HgCl_2$) aqueous solution, then washed three times with distilled sterile water to eliminate any remaining traces of the chemical. The sample was then blot dried once more. In a laminar-air-flow cabinet under aseptic conditions, these pieces were infected aseptically on autoclaved and cooled PDA media put in sterilised petri plates. The inoculation plates were kept at 26°C for 2 hours in a BOD incubator. After incubating for a week, a well-developed, contamination-free mycelial growth was discovered. The fungus was then transferred aseptically on PDA slants into test tubes using the hyphal tip isolation method. The test fungus was purified and maintained in PDA agar slants after 48–72 hours of observation and periodic sub-culturing.

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Growth of phylloplane mycoflora and causal pathogen in different culture media

Isolated phylloplane mycoflora and the causal pathogen were grown in the following cultural media to known the difference in their growth and sporulation in different media. Three replications were maintained for each phylloplane mycoflora and causal pathogen and the experiment was conducted in CRD and analyzed statistically.

Oat Meal Agar

Oat flakes- 40g, Agar- 20g, Distilled water-1000ml

Oat flakes were boiled in 400ml of distilled water for 20 minutes and then strained through a double layer of muslin cloth. Separately, agar was melted in 400ml of distilled water. These two solutions were thoroughly mixed, and additional distilled water was added to reach a final volume of 1000ml. The resulting media was subsequently sterilized in an autoclave at 15 psi pressure for 15 min.

Water Agar

Agar -20g

20 grams of Agar were suspended in 1000ml of distilled water. The mixture was heated to boiling to ensure complete dissolution of the medium. Subsequently, sterilization was performed by autoclaving at 15 lbs pressure (121^{0}C) for 15 minutes.

Richard's Synthetic Agar

Potassium Nitrate - 10g, Monopotassium dihydrogen Phosphate -5g, Magnesium Sulphate-2.5g, Ferric chloride- 0.02g , Sucrose- 15g. Dissolve 82.52 g 1000 ml of distilled water by heating until it is completely dissolved. Then, sterilize the mixture by autoclaving at 15 pounds per square inch (psi) and a temperature of 121°C for 15 minutes.

Malt extract Agar

Malt extract-30g, Mycological Peptone-5g, Agar-15g. Final pH (at 25°C) 5.4±0.2. Mix 50 g of the substance with 1000 ml of distilled water and let it soak for 15 minutes. Then, sterilize the mixture by autoclaving at 115°C and 10 pounds per square inch (psi) of pressure for 10 minutes.

Yeast Extract Agar

Yeast -5g, Glucose -10g, Agar -agar - 20g

Yeast agar powder of about 35g was suspended in 1000ml distilled water and heated to mix thoroughly. The medium was the sterilized in an autoclave at 15psi pressure with a temperature of 121.6ÚC for 15mins.

Potato Dextrose Agar

Peeled potato pieces - 200g, Agar agar -20g, Dextrose -20g, Distilled water -1000ml.

Initially, 200 g of peeled potatoes were cut and subsequently boiled in 400 ml of distilled water. Following that, 20 g of Agar were melted in another 400 ml of distilled water, and 20 g of Dextrose were introduced into the same container. These two solutions were thoroughly combined, resulting in a total volume of 1000ml. The mixture was then subjected to autoclaving at a pressure of 15 pounds per square inch (psi) for a duration of 15 minutes.

Sabouraud's Dextrose Agar

Dextrose -40g, Peptone -10g, Agar agar -15g, Distilled water- 1000ml

First, all the components were dissolved in 450ml of distilled water. In a separate container, 500ml of distilled water was used to dissolve Agar agar. These two solutions were then thoroughly mixed, and additional water was added to reach a total volume of 1000ml. The resulting mixture was subjected to autoclaving for 15 minutes under a pressure of 15 pounds per square inch (psi).

Statistical analysis

The experiments were cerried out under laboratory conditions using a completely randomized design (CRD) with three replications. Data obtained from the isolation of phylloplane mycoflora, growth characteristics of the leaf spot pathogen associated with *Solanum lycopersicum* L. were subjected to statistical analysis.

Results and Discussion

Identification, Isolation and purification of phylloplane mycoflora

Healthy leaf samples were collected and then cut into small fragments, which were subsequently placed on Potato Dextrose Agar (PDA) to cultivate various microorganisms present on the leaf surface. The microorganisms obtained were subjected to examination using a light microscope. To ensure purity, the microorganisms were isolated using the fungal tip method, and pure cultures were established for further investigation (Table 1).

Isolation, purification and Identification of Early blight pathogen of Tomato

Leaf samples that had been infected were collected and then transported to the laboratory with care. They were examined under a light microscope to detect the presence of the pathogen. Certain leaf samples were subjected to surface sterilization using a 0.1% solution of Mercuric chloride, while others were treated with a 1% Sodium hypochlorite solution. These sterilized samples were subsequently placed on Potato Dextrose Agar (PDA) plates to promote the growth of microorganisms. The pathogens were isolated using the fungal tip method, resulting in the creation of pure cultures intended for further examination (Table 2).

Growth of *Fusarium* sp.in different solid media

Fusarium sp was isolated from the phylloplane of tomato were grown in different media for knowing the best supportive media for growth of the fungus.

Table 1 : Colony characteristics of fungal isolates of Tomato phylloplane.

Isolate	Name of phylloplane mycoflora	Colour of the mycelium (upper side)	Colour of the mycelium (lower side)
1.	*Fusarium* sp.	White	Pale yellow
2.	*Aspergillus flavus*	Olive green	Cream to colourless
3.	*Aspergillus niger*	Black	Pale yellow to colourless

Table 2 : Colony characteristics of causal pathogen *Alternaria solani.*

Isolate	Name of pathogen	Colour of the mycelium (upper side)	Colour of the mycelium (lower side)
1.	*Alternaria solani*	Grey to olive green	Dark brown to black

Table 3 : Growth of *Fusarium* sp.in different solid media.

	Different solid media	Radial growth (mm)	Sporulation
T_1	Oatmeal Agar	80.46	+++
T_2	Water Agar	55.56	+
T_3	Malt Extract Agar	75.13	+++
T_4	Richard's Synthetic Agar	88.57	++
T_5	Yeast extract Agar	65.25	+
T_6	Potato Dextrose Agar	88.80	++++
T_7	Sabouraud's Dextrose Agar	83.46	++++
SEM(±)			**0.48**
CD (5%)			**1.46**

++++ Excellent, +++: Good, ++: Fair, +: Poor

Significant variations in the growth patterns of *Fusarium* sp *were* evident. The highest radial growth was observed on PDA (88.80mm), followed closely by Richard's Synthetic Agar (88.57mm), Sabouraud's Dextrose Agar displayed of 83.47mm, while Oatmeal Agar yielded radial growth of 80.74mm and Malt Extract Agar resulted in 75.14mm of growth. The least growth was noted on Water Agar (55.56mm) and Yeast extract Agar at 65.25mm. (Table 3).PDA and SDA exhibited excellent sporulation, while Oatmeal Agar and Malt Extract Agar showed fair sporulation, Richard's Synthetic Agar, and Sabouraud's Dextrose Agar displayed good levels of sporulation and Yeast Extract Agar and Water agar showed poor sporulation (Fig. 1).

a) *Fusaium sp.* on oat meal agar; b) *Fusaium sp.* on water agar; c) *Fusarium sp. on* malt extract agar; d) *Fusarium sp.* on richard synthetic Agar; e) *Fusarium sp.* on yeast extract agar; f) *Fusarium sp.* on PDA

Fig. 1 : Growth of *Fusarium* sp. in different media.

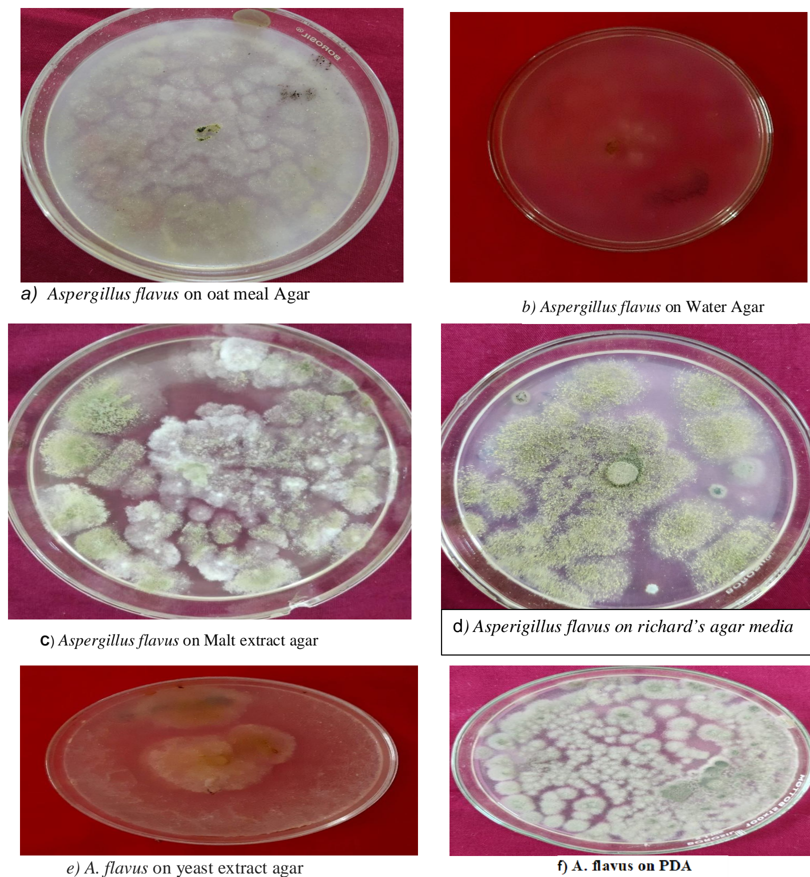
Growth of *Aspergillus flavus* in different solid media

Aspergillus flavus isolated from the phylloplane of tomato were grown in different media for knowing the best supportive media for growth of the fungus. Significant variations in growth patterns were notable in *Aspergillus flavus*, with the highest radial growth was observed on PDA (89.39mm), followed by Malt extract Agar (83.30mm), Richard's synthetic Agar (77.28mm),

Table 4 : Growth of *Aspergillus flavus* in different solid media.

Treatment	Different solid media	Radial growth (mm)	Sporulation
T_1	Oatmeal Agar	71.59	++
T_2	Water Agar	14.61	+
T_3	Malt Extract Agar	83.30	+++
T_4	Richard's Synthetic Agar	77.28	+++
T_5	Yeast Extract Agar	58.7	+
T_6	Potato Dextrose Agar	89.39	++++
T_7	Sabouraud's Dextrose Agar	74.45	+++
SEM (±)		**0.38**	
CD (5%)		**1.18**	

++++ Excellent, +++: Good, ++: Fair, +: Poor



a) *Aspergillus flavus* on oat meal Agar

b) *Aspergillus flavus* on Water Agar

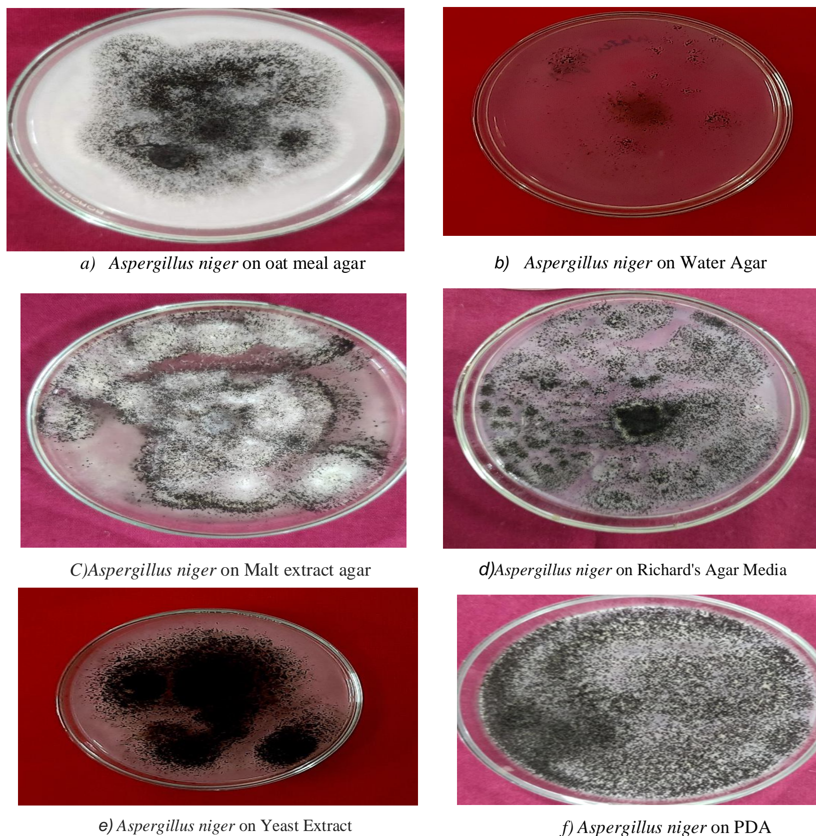
c) *Aspergillus flavus* on Malt extract agar

d) *Asperigillus flavus on richard's agar media*

e) *A. flavus* on yeast extract agar

f) A. flavus on PDA

Fig. 2 : Growth of *Aspergillus flavus* in different media.



a) *Aspergillus niger* on oat meal agar

b) *Aspergillus niger* on Water Agar

C)*Aspergillus niger* on Malt extract agar

d)*Aspergillus niger* on Richard's Agar Media

e) *Aspergillus niger* on Yeast Extract Agar

f) *Aspergillus niger* on PDA

Fig. 3 : Growth of *Aspergillus niger* in different solid media.

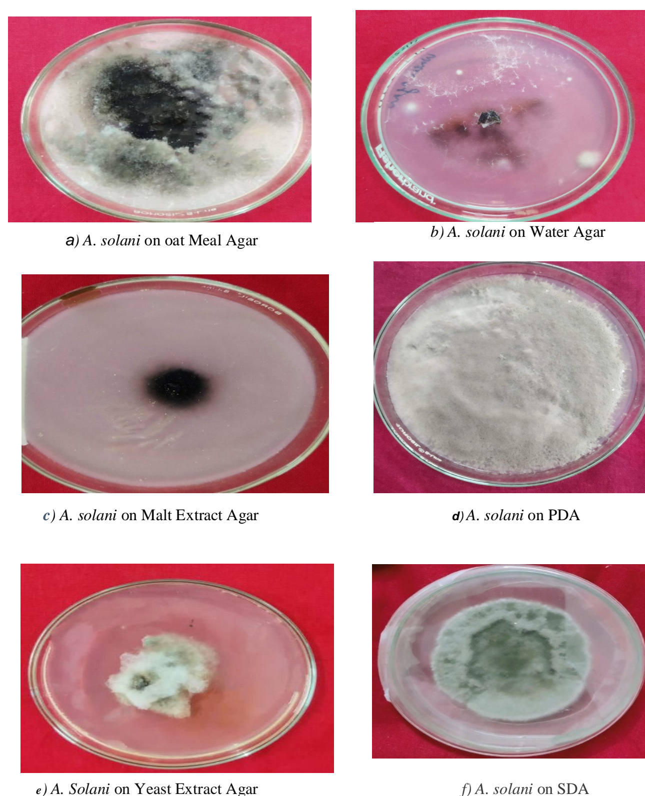
Sabouraud's Dextrose Agar (74.45mm), Oatmeal Agar (71.59mm), Yeast Extract Agar (58.7mm) The least radial growth was observed on Water Agar. (14.61mm). (Table -4). PDA exhibited excellent sporulation, while, Richard's Synthetic Agar, Malt Extract Agar and Sabouraud's Dextrose Agar showed good sporulation. Oatmeal Agar displayed fair sporulation, while Water Agar and Yeast extract Agar exhibited poor sporulation (Fig. 2).

Growth of *Aspergillus niger* in different media

Aspergillus niger isolated from the phylloplane of tomato were grown in different media for knowing the best supportive media for growth of the *Aspergillus niger*. Significant Variations in growth patterns were noted in *Aspergillus niger*. The highest radial growth was observed in Richard's Synthetic Agar (89.97mm), followed by PDA (88.50), Malt Extract Agar (83.57mm), Oatmeal Agar (78.47mm), Yeast Extract agar (82.57mm) and the growth of Aspergillus niger in Richard's Synthetic Agar and PDA were at par showing similar growth pattern of 89.97mm and 88.50 mm respectively. Water Agar (19.31mm) exhibited the least radial growth at 65.39mm. (Table 5). Exceptional sporulation was observed in PDA and Richard's Synthetic Agar. Good sporulation was noted in Oatmeal Agar, Malt Extract Agar, Yeast Extract Agar. Sabouraud's Dextrose Agar displayed fair sporulation and Water Agar exhibited least sporulation (Fig. 3).

Growth of causal pathogen i.e., *Alternaria solani* in different culture media

Significant variations in growth patterns were notable in *Alternaria solani* with the highest radial growth occurring on PDA (88.28mm), followed by Sabouraud's Dextrose Agar (86.35mm), Malt extract Agar (84.69mm), Oatmeal Agar (79.78mm), Yeast Extract Agar (46.88mm), Richard's synthetic Agar (36.32mm). The least radial growth was observed on Water Agar. (12.15mm). (Table 6). PDA and



a) *A. solani* on oat Meal Agar
b) *A. solani* on Water Agar
c) *A. solani* on Malt Extract Agar
d) *A. solani* on PDA
e) *A. Solani* on Yeast Extract Agar
f) *A. solani* on SDA

Fig. 4 : Growth of causal pathogen i.e., *Alternaria solani* in different solid media.

Table 5 : Growth of *Asperigillus niger* in different media.

Treatment	Different solid media	Radial growth (mm)	Sporulation
T_1	Oatmeal Agar	78.47	+++
T_2	Water Agar	19.31	+
T_3	Malt Extract Agar	83.57	+++
T_4	Richard's Synthetic Agar	89.97	++++
T_5	Yeast Extract Agar	82.57	+++
T_6	Potato Dextrose Agar	88.50	++++
T_7	Sabouraud's Dextrose Agar	65.38	++
SEM (±)		**0.50**	
CD (5%)		**1.52**	

++++ Excellent, +++: Good, ++: Fair, +: Poor

SDA exhibited excellent sporulation, while, Malt Extract Agar showed good sporulation. Oatmeal Agar displayed fair sporulation, while, Richard's Synthetic Agar Water Agar and Yeast extract Agar exhibited poor sporulation (Fig. 4).

The best growth medium for *Fusarium* sp. was determined to be potato dextrose agar (PDA) with a diameter of 88.80mm. Richard's synthetic agar, with a diameter of 88.57mm, was also found to be suitable for its growth. Bhagwat (1969) reported that PDA was the most effective medium for promoting mycelial growth and sporulation of *Fusarium*. sp. Similarly, Moura *et al.* (2020) observed that both Potato dextrose agar and Richard's synthetic agar were the most favourable choices for stimulating mycelial growth of this fungus.

Table 6 : Growth of *Alternaria solani* in different media.

Treatment	Different solid media	Radial growth (mm)	Sporulation
T_1	Oatmeal Agar	79.78	++
T_2	Water Agar	12.15	+
T_3	Malt Extract Agar	84.69	+++
T_4	Richard's Synthetic Agar	36.32	+
T_5	Yeast Extract Agar	46.88	+
T_6	Potato Dextrose Agar	88.28	++++
T_7	Sabouraud's Dextrose Agar	86.35	++++
SEM (±)		**0.83**	
CD (5%)		**2.54**	

++++ Excellent, +++: Good, ++: Fair, +: Poor

The fungus *Aspergillus niger* displayed its most significant growth on Richard's synthetic agar, measuring 89.97mm, and closely followed by Potato dextrose agar, with a growth of 88.50mm. These findings align with those reported by Pathak in 1993, as well as the results obtained by Amrita and Richa in 2014.

Aspergillus flavus, when cultivated on various solid media, exhibited exceptional sporulation and the highest growth when placed on potato dextrose agar (PDA) with a diameter of 89.39mm. These findings align with the research findings of Raper and Fennel from 1965.

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