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Effect of different media on growth and cultural characterization of *Alternaria alternata* causing leaf spot of chilli

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Abstract
Alternaria is known for its most noticeable feature having concentric rings in the centre of the spots on leaves. In this present investigation, the morphological and cultural Charactization of the fungus was identified by performing the isolation of the pathogen from the diseased samples of the chilli leaves and establishing it's identification through the pathogenicity test by spore suspension method and the results revealed that the identifying fungus showing the symptoms start appearing after seven to nine days of inoculation with small, circular brown to black spots surrounded by yellow hallow on chilli leaves. In severe cases, concentric rings appeared in the centre of the spots, the fungus was identified as *Alternaria alternata*. The radial growth pattern of Alternaria were observed on different temperatures, pH and humidity levels as well as also observed on different synthetic and non – synthetic media and the present study identified that the maximum radial growth of the fungus was observed at 30 °C (71 mm) temperature followed by 25 °C (69.5 mm) and the minimum growth was observed at 15 °C (26.5 mm) temperature. For the maximum growth of the fungus, the best pH observed was 6.5 (68.5 mm) followed by 6.0 (61 mm) and the minimum growth was observed at pH 4.0 (47 mm).The best relative humidity on which the pathogen exhibits maximum radial growth was 80 per cent (78.16 mm) followed by 90 per cent (74.66 mm) and the minimum growth was observed at 60 per cent (68.33 mm).The best synthetic media on which the maximum radial growth was observed is Richard's agar (86.83 mm) and the best non-synthetic media on which the maximum radial growth was observed is Oatmeal agar media (75.5 mm). Thus by knowing the best temperature, pH and humidity levels as well as getting the best media other than PDA for the maximum growth of *Alternaria alternata*.

Keywords: Media, cultural, characterization, *Alternaria alternata*, chilli

Introduction

Chilli (*Capsicum annuum* L.) is well known for its aroma, pungency and medicinal value. It is a perennial herbaceous plant that belongs to the family Solanaceae having 2n = 24 chromosome number. Both vegetarian and non-vegetarian dishes considered chilli as the most important ingredient for its taste and flavour. According to a report published on Indiastat.com by Professor Jayashankar Telangana State Agricultural University (Agricultural Market Intelligence Centre) showing the area, production, and productivity of chilli in India (2020-30th June 2021), so as per the report, the chilli covered 7.43 lakh ha area with 19.14 lakh tonnes production and productivity of 2576 kg/ha and for this reason it makes India the world's largest producer, consumer and exporter of chilli. According to Spice Board India report (2019-2020 Est.), chilli growing states in India with their production (Lakh Tonnes) are: Andhra Pradesh (6.60 lakh tonnes), Telangana (3.28 lakh tonnes), Madhya Pradesh (2.18 lakh tonnes), Karnataka (1.80 lakh tonnes) and West Bengal (1.04 lakh tonnes). The most noticeable character of the fungus is the development of the concentric rings in the centre of the spots on leaves. Symptoms appear on different parts of the plants such as on leaves, twigs and fruits. The presence of brown to black necrotic lesions on older leaves, ranging one to five mm wide and producing a bull's eye appearance of concentric rings. On getting favourable conditions, several lesions coalesce and thus results in leaf drop. Usually, the spots formed by the *Alternaria* are surrounded by a chlorotic halo.

Material and Methods

Isolation of Pathogen

Method of preparing culture media-

Under *in vitro* conditions, a potato dextrose agar (PDA) medium was used to isolate the target pathogen.

Way to make PDA, took 200 g of peeled potatoes cut in small slices and then boiled in 500 ml of distilled water to make potato extract. With the help of muslin cloth strained out the potato extract in a beaker. 20 g agar-agar was taken in 500 ml distilled water and melts it properly. When it melts properly, add 20 g dextrose stir it, then mix it with the potato extract properly and make up the volume 1000 ml by adding distilled water in it. PDA was ready to pour into flasks and then plugged with the non-absorbent cotton and sterilized in an autoclave.
For preparing slants of PDA – took culture tubes and pour 4-5 ml of PDA in it under aseptic condition inside the laminar airflow chamber and then after plugging with the non-absorbent cotton, sterilized in an autoclave. After autoclaving, the culture tubes were placed in a slanting position on a wooden support and allowed them to solidify under aseptic conditions. And then can be stored in a refrigerator.

Isolation
Diseased samples collected from different areas during the season and isolated in the laboratory. Firstly collected diseased samples were washed thoroughly under the tap water and then cut into small pieces 2-4 mm in size with the help of a sterilized blade in such a way that the sample contained a 50 per cent healthy portion as well as a 50 per cent diseased portion. The surface of the pieces was sterilized by using 1 per cent Sodium hypochlorite solution for 30 seconds to 1 minute, then finally washed well with the three changes of sterilized distilled water and to remove excess water then pieces were placed on blotter paper. With the help of a sterilized inoculating needle place the sample pieces on petri-plates containing potato dextrose agar medium under the aseptic conditions in the laminar airflow chamber. Three replications were made by placing three pieces on PDA media on each plate. Inoculated petri plates were kept in an incubator at 25°±2 °C and examined at frequent intervals to check the growth of the target fungal pathogen.

Purification and maintenance of the target pathogen
By using the single spore method (Toussoun and Nelson, 1976) [26], the purification of the culture was made. At regular intervals, sub-culturing was done. The pure culture was maintained in slants and stored at 4 °C temperature in the refrigerator.

Identification of the isolated pathogen
With the help of available literature and the cultural and morphological features, the identification of the isolated fungus was done. It starts by identifying the colony characters on the PDA medium on which the target isolated pathogen was grown and incubated at 25° ± 2 °C and examine the growth of the pathogen after 7 days. By observing 20-30 days old cultures, the morphology of the conidia was studied properly.

Pathogenicity test for the isolated pathogen
The technique used is the inoculums spore suspension method on the detached leaves of chilli under *in vitro* as well as under natural conditions on leaves of the chilli plant. Ten days old culture was used for making inoculums spore suspension. The spraying was done with the help of an atomizer. By using polythene bags the humidity was maintained around the chilli plant. Un- inoculated pots were served as control. At regular intervals, the examination was continued till the expression of the typical symptoms appeared. Compared the symptoms that appeared with the symptoms produced by the mother cultures to confirm its pathogenicity.

Effect of different temperatures on radial growth of *Alternaria alternata*
Under the study, five levels of temperature were used to determine their effect on radial growth of *A. alternata*. The temperatures of BOD were calibrated at 15 °C, 20 °C, 25 °C, 30 °C and 35 °C. A five mm disc was taken from the ten-day-old culture and was placed in the centre of the Petri-plates containing @ 20ml PDA under the aseptic conditions inside the laminar air flow chamber and then kept in BOD incubator at different temperatures. Three replications were made. For each temperature radial growth of the colony was recorded after 48 hrs, 96 hrs, 144 hrs and 192 hrs intervals.

Effect of pH levels on radial growth of *A. alternata*
Studies on the effect of pH levels on characters of *A. alternata* was undertaken on PDA media at intervals of 48 hrs, 96 hrs, 144 hrs and 192 hrs. During the investigation, five levels of pH were used *viz.* 4.0 pH, 6.0 pH, 6.5 pH, 7.0 pH and 7.5 pH at an interval of 0.5 were adjusted with citrate and phosphate buffer. N/10 HCl and N/10 NaOH were used for calibrations of pH. Plates were incubated at 25° ± 2 °C for eight days. Radial growth of the colony was recorded at different pH.

Determining the effect of different relative humidity levels on radial growth of *A. alternata*
To study the effect of relative humidity on radial growth of *Alternaria alternata*, five different levels were used that is, 60 per cent, 70 per cent, 80 per cent, 90 per cent and 100 per cent. These different humidity levels were maintained by using the concentrated sulphuric acid and sterilized distilled water in different proportions in glass desiccators as per the method suggested by Buxton and Mellanby (1934) [2].

The composition of the acid solution was as follows

RH (%)	Stock solution (ml) of H_2SO_4	Distilled water (ml)
60	120.2	79.8
70	132.4	67.6
80	145.8	54.2
90	166.6	33.4
100	200	0

Petri plates containing PDA medium were inoculated with a five mm disc from seven days old culture of *A. alternata* with the help of a sterilized corn borer. Inoculated Petri plates were immediately accommodated in the glass desiccators containing a mixture of sulphuric acid and distilled water in required proportion and incubated at room temperature for seven days. Radial growth of the colony was recorded after 48 hrs, 96 hrs, 144 hrs and 192 hrs intervals.

Effect of different media (non-synthetic and synthetic) on the growth of *Alternaria alternata*
Different synthetic and non-synthetic media were used to study the growth of *A. alternata*. Total eleven media were used, out of which eight were non- synthetic media *viz.* Oatmeal agar, Potato dextrose agar, Carrot agar, Radish agar, Chilli leaves agar, Chilli fruit agar, Tomato leaves agar, Tomato fruit agar and three were synthetic media *viz.*

Richard's agar, Rose Bengal agar and Czapek's- Dox agar were prepared to undertake the investigation. Prepared media was then subjected for sterilization in an autoclave. After autoclaving, the media were poured into the sterilized Petri plates @ 20 ml/plate and allowed them to solidify under the aseptic condition. For inoculation, ten-day-old culture was used and a 5 mm mycelial disc was inoculated in the centre of each Petri plate. These plates were then incubated at 25°±2°C till the complete colonization. Three replications were made. Radial growth of the colony was recorded after 48 hrs, 96 hrs, 144 hrs and 192 hrs intervals. Observations were recorded for the best media on which the fungus's growth was maximum.

Results

Isolation and Identification of the target pathogen (*A. alternata*)

The fungal colony culture was initially white, cottony, and abundant in aerial mycelium, but it progressively turned grey. With aerial mycelium, the old culture appeared entirely grey, and clear concentric rings formed on the PDA medium. Conidiophores ranged simple to branched, and appeared singly. Conidia were seen in lengthy chains on conidiophores, with strong walls, beaked edges, and had transverse and longitudinal septation and were golden to brown in appearance. The fungus was identified as *Alternaria alternata* based on colony characteristics and morphological characteristics of conidiophores and conidia (Devappa and Kumar, 2016) [3].

Purification of the Pathogen

The culture obtained was purified once after the pathogen identity was confirmed. Periodic sub-culturing on PDA slants was done to keep the pure culture. Throughout the investigation, this pure culture was employed.

Pathogenicity

The pathogenicity of *A. alternata* was confirmed using the inoculum spore suspension method on healthy chilli plants in the field and on detached chilli leaves in the lab by inoculating with the same inoculum spore suspension.

Symptomatology

Under natural condition, the disease symptoms appeared on the inoculated leaves as a small, circular necrotic patch with concentric rings. *Alternaria* causes little reddish-purple spots on older leaves, with brown or dark spots as the most common symptom. These spots began to grow with an irregular edge, stayed brown, and were surrounded by yellow hallow. The spotted leaves withered quickly, and the plant died.

Effect of temperature on radial growth of *Alternaria alternata*

The maximum radial growth was found at 30 °C (71 mm), followed by 25 °C (69.5 mm), and 35 °C (65.5 mm) whereas, the minimum growth (26.5 mm) was observed at 15 °C.

Table 1: Effect of temperature on radial growth of *A. alternata*

Temperature (°C)	Radial growth (mm) * after hours			
	48 hrs	96 hrs	144 hrs	192 hrs
15°	8.33	18.67	24.67	26.5
20°	14	20.5	26.67	30.5
25°	20.5	24.67	48	69.5
30°	20.33	24.67	48.5	71
35°	20	23.67	45.5	65.5
S.Em.±	0.357	0.325	0.247	0.289
C.D at 5%	1.141	1.037	0.789	0.921
C.V.	3.722	2.508	1.107	0.951

* Mean of three replications

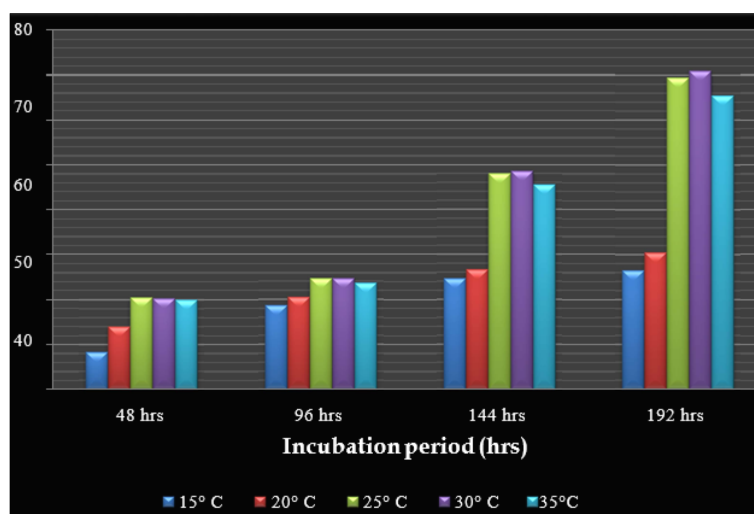

Fig 1: Radial growth (mm) of *Alternaria alternata* at different temperatures

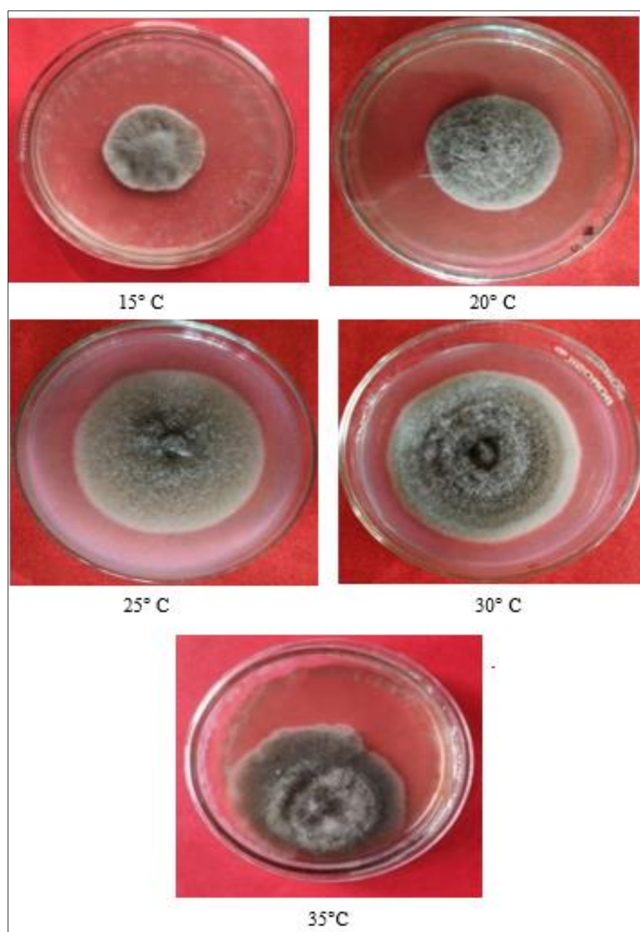

Plate 1: Effect of temperature on mycelial growth of *A. alternata* (after 192 hrs)

Effect of pH levels on radial growth of *A. alternata*

The findings given in Table 2 demonstrated that the maximum radial development was seen in *A. alternata* at pH 6.5 (68.5 mm), followed by pH 6.0 (61 mm), and pH 7.5 (50 mm). At pH 4.0, the minimum amount of growth was observed (47 mm).

Table 2: Effect of pH levels on radial growth of *A. alternata*

pH	Radial growth (mm) *after hours			
	48 hrs	96 hrs	144 hrs	192 hrs
4.0	32	40	45	47
6.0	34	50.67	59.5	61
6.5	35	48	60.5	68.5
7.0	33.5	42	48	49.33
7.5	35	46.33	49.5	50
S.Em.±	0.307	0.307	0.289	0.269
C.D at 5%	0.921	0.981	0.921	0.858
C.V.	1.475	1.172	0.952	0.844

* Mean of three replications

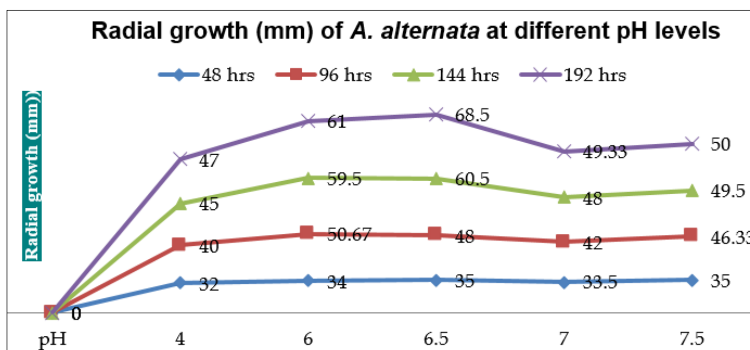

Fig 2: Radial growth (mm) of *A. alternata* at different pH levels

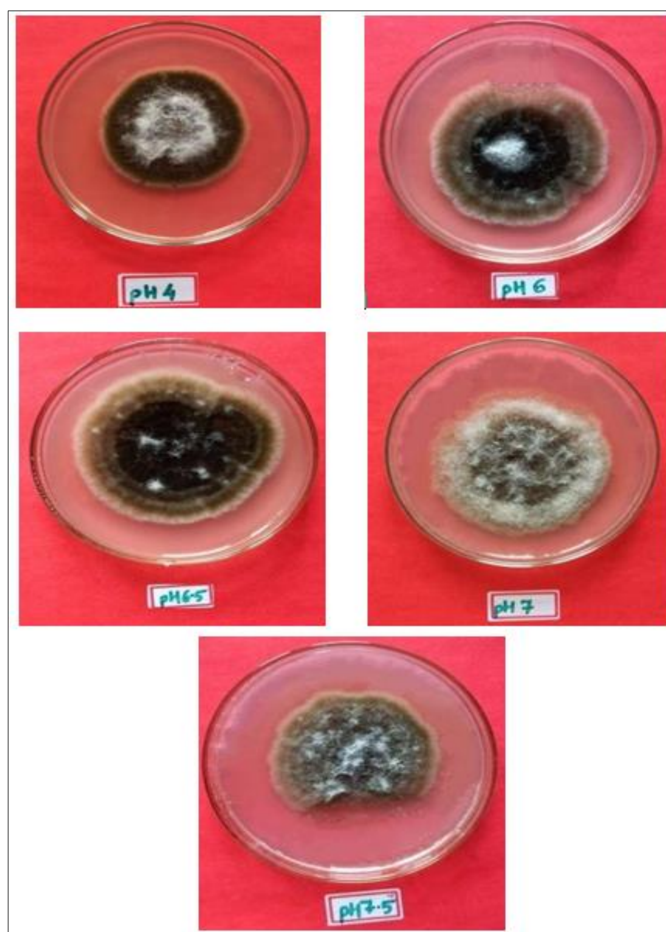

Plate 2: Effect of pH levels on mycelial growth of *A. alternata* (after 192 hrs)

Effect of relative humidity on radial growth of *A. alternata*
The best relative humidity per cent for *A. alternata* on which it exhibits the maximum radial growth was 80 per cent (78.16 mm), followed by RH 90 per cent (77 mm), and RH 70 per cent (74.83 mm), as shown in Table 3 and the minimum radial growth 68.33 mm was observed at RH 60 per cent.

Table 3: Effect of relative humidity on radial growth of *A. alternata*

Relative Humidity (RH) %	Radial growth (mm) * after hours			
	48 hrs	96 hrs	144 hrs	192 hrs
60%	16.83	38.67	53.83	68.33
70%	18	40.50	54.17	74.83
80%	21.33	47.33	57.5	78.16
90%	22	45.33	58.33	77
100%	19.83	43.17	54.17	74.66
S.Em.±	0.582	0.592	0.679	0.373
C.D at 5%	1.858	1.888	2.167	1.190
C.V.	5.189	2.402	2.103	0.861

Mean of three replications

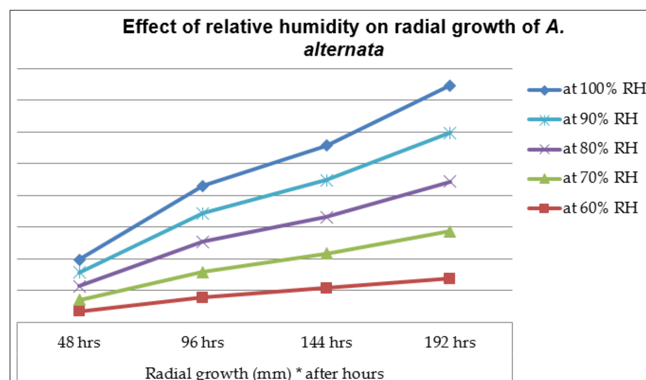

Fig 3: Radial growth (mm) of *A. alternata* at different relative humidity levels

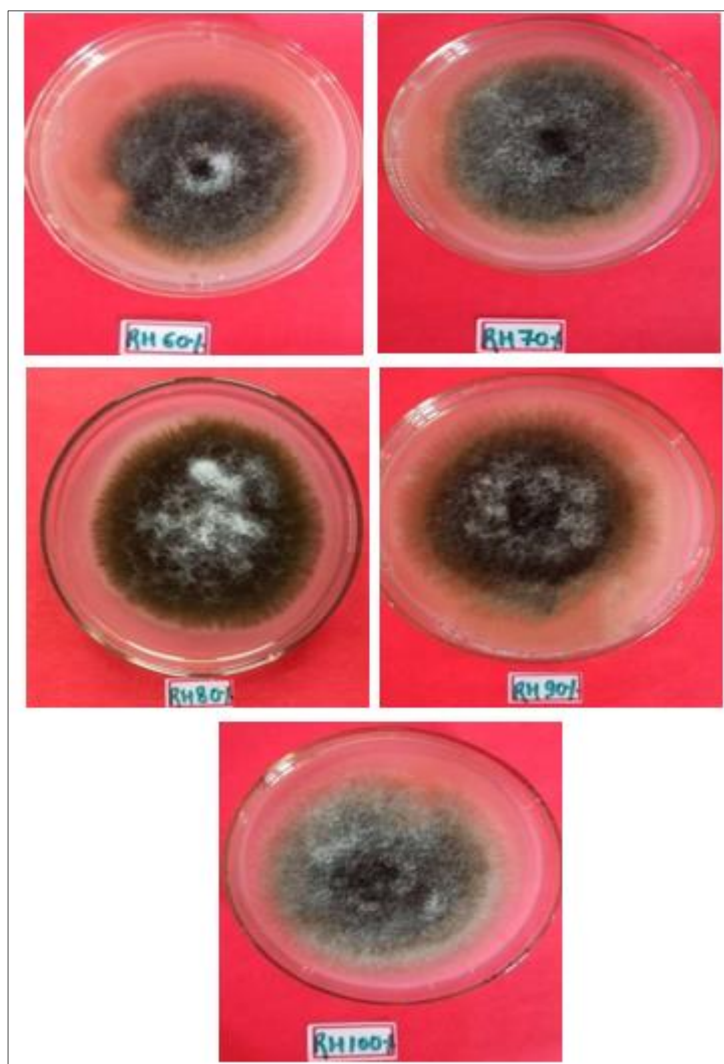

Plate 3: Effect of relative humidity on mycelial growth of *A. alternata* (after 192 hrs)

Effect of different media on growth of *A. alternata*

The mean radial growth of *A. alternata* was significantly different on all media tested, in synthetic media, the fungus showing the maximum radial growth on Richard's agar (86.83 mm), while in case of non-synthetic media, the fungus showing the maximum radial growth on Oatmeal agar media (75.5 mm), followed by potato dextrose agar (75 mm), and tomato fruit agar (74.43 mm). On tomato leaf agar, there was very little fungal development (32 mm). With an increasing incubation period, radial growth also increased. Between 144 and 192 hours of incubation, the maximum growth was observed on Richard's agar and Oatmeal agar. Colony characteristics vary depending on the media examined; colony borders were smooth in all synthetic media, and colony texture was fluffy in all media except Rose Bengal agar, which had appressed colonies.

Table 4: Effect of different media on radial growth of *A. alternata*

Media	Radial growth (mm) *after hours			
	48 hrs	96 hrs	144 hrs	192 hrs
Oat meal agar	27	35.6	56.23	75.5
Potato dextrose agar	19.67	30.83	46.6	75
Tomato fruit agar	22.7	32.57	41.77	74.43
Carrot agar	20.1	28.83	45.17	62.23
Radish agar	21.93	32.97	48.23	57.1
Chilli fruit agar	18.5	28.10	35.83	47
Chilli leaf agar	14.33	17.8	25.77	43.13
Tomato leaf agar	17.17	26.63	30	32
Richard’s agar	25.83	32.5	48.67	86.83
Rose bengal agar	22	26.83	45.33	72
Czapek’s Dox agar	19	22.83	48.50	85.5
S.Em.±	0.350	0.330	0.292	0.533
C.D at 5%	1.033	0.973	0.861	1.572
C.V.	2.920	1.990	1.177	1.453

* Mean of three replications

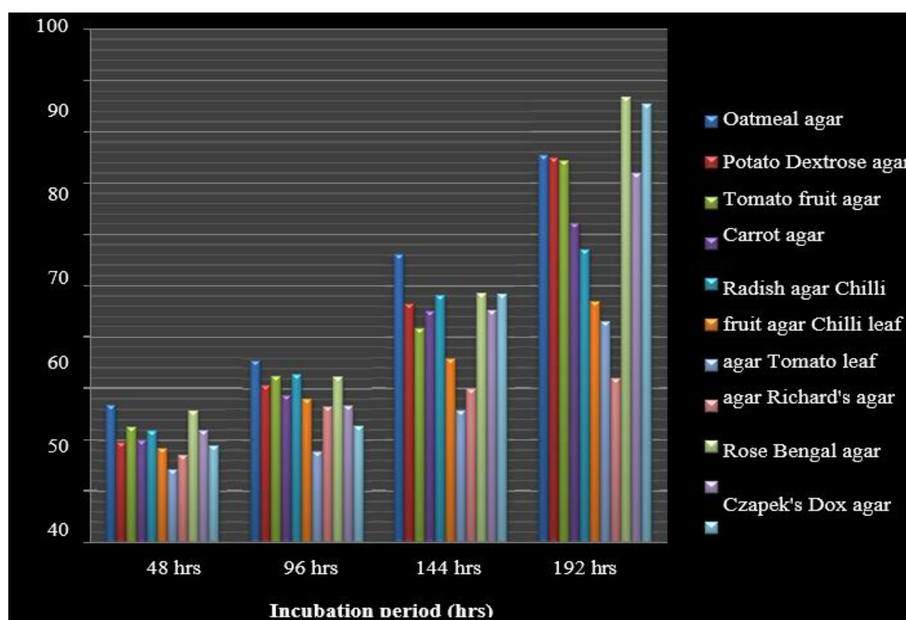

Fig 4: Radial growth (mm) of *A. alternata* on different non-synthetic and synthetic media

Plate 4: Effect of different natural media on mycelia growth of *A. alternata* (after 192 hrs)

Plate 5: Effect of different synthetic media on mycelial growth of *A. alternata* (after 192 hrs)

Discussion

Isolation of the pathogen

Using PDA as a basal medium, the pathogen *A. alternata* was isolated from infected chilli leaves. The fungal colony culture was initially white, cottony, and abundant in aerial mycelium, but after a few more days of incubation, it turned into an olivaceous green colour. Conidiophores ranged simple to branched, and arose singly bearing muriform conidia with transverse and longitudinal septation, thick walls, and brown colour. The test fungus was then purified, maintained on agar slants, and kept at 4 °C in the refrigerator. The fungus was identified as *Alternaria alternata* based on cultural, morphological, and symptomatological characteristics. This suggests that *A. alternata* is linked to chilli leaf spot. These findings are largely consistent with those of Devappa and Kumar (2016) [3], who isolated fungus *A. alternata* from diseased chilli leaves on PDA medium.

Identification of the pathogen

On PDA, the fungus produced profuse mycelial growth. It turned grey - brownish, multicelled, septate, and irregularly branched. Initially, hyphae were thin and hyaline, but as they expanded, they became slightly thicker. Conidia were borne in chains, muriform, and ranged in colour from light olivaceous to dark brown.

Several workers have identified *A. alternata* as the cause of the chilli leaf spot. Ginoya and Gohel (2015) [5] examined the size of conidia (length and breadth), beak length, and several

septa (transverse and longitudinal) from a 15-day-old culture under high power magnification to identify morphological features of distinct isolates of *A. alternata*.

According to Devappa and Kumar (2016) [3], conidiophores were simple or branched, arose singly and ranged in colour from yellow to brown. Conidia were seen in lengthy chains on conidiophores, with strong walls, beaked edges, and brown colour. The fungus was identified as *Alternaria alternata* based on colony characteristics and morphological characteristics of conidiophores and conidia.

Pathogenicity test of target pathogen (*Alternaria alternata*)

The pathogenicity test of *A. alternata* was demonstrated on healthy chilli plants in their natural environment as well as also under *in vitro* condition on detached chilli leaves using the spore suspension method. Initial signs on leaves with a tiny and circular necrotic patch appeared after seven to nine days of inoculation. Concentric rings are common on leaves in severe infections. Brown or dark spots on older leaves are the most noticeable symptom.

Several researchers, including Khodke and Gahukar, 1993 [10], and Thippeswamy *et al*., 2007 [24], identified *A. alternata*, the cause of chilli leaf spot, and demonstrated its pathogenicity on chilli seedlings.

Devappa and Kumar (2016) [3] demonstrated pathogenicity by inoculating the chilli plants though *Alternaria* spore suspension and also maintaining control without inoculation. Alternaria leaf spot symptoms appear as a tiny, circular necrotic patch on the inoculated leaves, seven to nine days after inoculation. These spots began to grow in size with an irregular edge, and they remained brown with a yellow halo. This discovery is consistent with that of Atia and Tohamy (2004) [1], Mangala *et al*. (2006) [12], and Li *et al*. (2011) [16], all of whom found pathogenicity in *A. alternata*.

Symptomatology

The symptomatology of the chilli leaf spot produced by *A. alternata* was studied in detail. *Alternaria* spp. causes little reddish-purple dots on leaves, with brown or dark spots on older leaves being the most common symptom. These patches began to grow with an irregular edge and stayed brown with a yellow halo, eventually withering and killing the plant.

Sreekantiah *et al*. (1973) [23] observed that infection occurred on treated chilli plants in 7 days, with early-stage circular-shaped spots up to 1 cm on leaves, which enlarge and turn brown into irregular sunken patch with a dark brown edge. Spots were more prevalent near the leaf's margins and resembled those found on fruits.

Devappa and Kumar (2016) [3], inoculated chilli leaves with spore suspension made in sterile distilled water and sprayed individually on the plants. The symptoms appeared on the lower side of the leaves first. A tiny, circular necrotic patch appeared after seven to nine days of inoculation as a sign of Alternaria leaf spot. These spots began to grow with an uneven edge and remained brown with a yellow halo. Later on, the size and quantity of spots grew larger, and the spots began to consolidate. For validation of the fungus under research, the isolate was compared to the original culture of *Alternaria alternata*.

Effect of temperature on radial growth of *Alternaria alternata*

The fungus grew well at temperatures ranging from 20 °C to 35 °C, with 30 °C being the best temperature for its growth, followed by 25 °C.

Temperature plays a vital role among the external elements, according to the past studies conducted by authors such as Devappa and Kumar (2016) [3]. The optimum temperature range for mycelial growth and spore germination of *A. alternata* was determined to be between 25 ° and 30 °C. Waghunde and Patil (2010) [27] found similar results, states that 25 °C was the optimal temperature for mycelial development and sporulation of *A. alternata*.

Guo *et al*. (2011) [7] found that *A. alternata* grows best at temperatures between 25 °C and 30 °C, while Hubballi *et al*. (2010) [8] investigated the influence of various temperatures on the growth of *A. alternata* under *in vitro*.

According to the results of the experiment, the maximum mycelial growth was reported at 25°-30 °C temperatures (range from 69.5 – 71 mm after 192 hours) while assessing the mean radial growth of *A. alternata* at different temperatures.

Effect of pH levels on radial growth of *A. alternata*

The role of pH in mycelial growth and development at varied incubation intervals was studied. pH ranging from 6.0 to 7.5 aided *A. alternata* radial growth. Its colonisation and sporulation have been documented by Ramjegathesh and Ebenezar (2012) [19]; Madhavi *et al*. (2012) [11]; Hubbali *et al*. (2010) [8]; Jash *et al*. (2003) [9]; Saeed *et al*. (1995) [20]; Mohapatra *et al*. (1978) [14]; Samuel *et al*. (1972) [21].

According to the results of the experiment, the ideal pH range for *A. alternata* radial growth was 6.0 to 7.5. Mean radial growth of *A. alternata* was found to be maximum at a pH of 6.0 -6.5 (ranging from 61- 68.5 mm after 192 hrs).

Effect of relative humidity levels on radial growth of *A. alternata*

Ghewande (1986) [4] discovered that temperatures between 25 °C and 29 °C, with a relative humidity of 87 per cent, were more favourable for the radial development of Alternaria leaf spot of groundnut induced by *A. alternata*.

Patel and Patel (1991) [18] found that a temperature range of 25°-40 °C and high relative humidity favoured the growth of *A. alternata*- causing tomato rots in the open market. *A. alternata* caused significant rot disease in pomegranates, according to Singh and Majumdar (2000) [22]. At a temperature of 25 °C and relative humidity of 90 per cent, the rotting was at its peak.

Observation of mean radial growth of *A. alternata* at different relative humidity levels shows that maximum growth occurs at 80 per cent-90 per cent RH (ranging from 78.16 – 77 mm after 192 hrs).

Effect of different media on growth of *A. alternata*-

Eleven different solid (synthetic and non-synthetic) media were chosen to evaluate their effect on radial growth of *A. alternata*. The results show that the Richard's agar medium (86.83 mm) had the highest radial growth of all the synthetic and non-synthetic media, and it outperformed them all.

A. alternata was also grown best on Czapek's Dox agar (85.5 mm) and Oatmeal agar (75.5 mm) media. Potato Dextrose agar (75 mm), Tomato Fruit agar (74.43 mm), Rose Bengal agar (72 mm), Carrot agar (62.23 mm), Radish agar (57.1 mm), and Chilli fruit agar(47 mm) were the next best in order of merit. Tomato leaf agar (32 mm) and Chilli leaf agar

(43.13 mm) had shown the minimum radial growth of *A. alternata*.

According to the results of the experiment, after 192 hours, the maximum mean radial growth was observed in Oatmeal agar medium (75.5 mm), followed by PDA (75mm) in non-synthetic media, and in synthetic media, the maximum growth was found in Richard's agar (86.83mm) followed by Czapek's Dox agar (85.5 mm).

Richard's agar medium supported great growth and sporulation of *A. alternata*, according to Patel (1993) [17], and he also discovered excellent sporulation on both solid and liquid media containing tomato leaves extract.

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