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Cultural Analysis and Growth Kinetics of *Colletotrichum circinans* Causing Onion Smudge

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Abstract

The present investigations were conducted during October, 2021 to April, 2022 under laboratory conditions in the Department of Plant Pathology, College of Horticulture and Forestry, Neri, Hamirpur. The test fungus *C. circinans* was isolated on potato dextrose agar from symptomatic onion bulb. Colour of mycelium was cremish white and growth was observed to be dense, cottony and suppressed. Pathogenicity test was conducted by incision method on the onion bulbs inoculated with the isolated pathogen resulted in appearance of anthracnose symptoms on inoculated part of the bulb. Different nutrient media, temperature regimes and pH levels were evaluated for the growth of *Colletotrichum circinans* causing onion smudge. Among six different nutrient media evaluated, potato dextrose agar gave maximum mycelial growth (90.00 mm) and growth rate (0.70 mm h^{-1}) with pure white, cottony and fluffy mycelium exhibiting ray growth pattern after 120 hours of incubation and minimum mean mycelial growth (23.81 mm) as well as minimum growth rate (0.35 mm h^{-1}) was recorded in Richard's agar. Out of five different temperature regimes, 30°C was observed to be the best temperature for the mycelial growth (90.00 mm) with maximum growth rate (0.71 mm h^{-1}) with white, strandy and cottony mycelial growth exhibiting ring pattern after 120 hours of incubation and minimum (5.00 mm) was recorded at 15°C. Among seven pH levels, pH 7.0 was observed to be the best pH for supporting the maximum mycelial growth (90.00 mm) and growth rate (0.70 mm h^{-1}) with transparent at centre and white at margins exhibiting cottony growth with ray pattern after 120 hours of incubation and mean minimum mycelial growth (31.23 mm) was recorded at pH 5.0. The growth rate of the fungus was highest between 48–72 h of incubation in different experiments.

Keywords: *Colletotrichum circinans*, onion smudge, nutrient media, mycelial growth

1. Introduction

The onion (*Allium cepa* L.) belonging to family Alliaceae is either biannual or perennial (depending on the cultivar) in nature and has a pungent odour when crushed. Since ancient times, the onion has been valued as a food and a medicinal plant. It is the most frequently cultivated and consumed vegetable, second only to the tomato, which is familiar to most cultures and consumed worldwide (Anonymous, 2012). Due to its highly valued flavour, aroma, unique taste and the medicinal properties of its flavour compounds, it is commonly known as "Queen of the kitchen" Griffiths et al. (2002).

Onion production and marketing are affected by more than a dozen diseases caused by fungi, bacteria and viruses (Kiehr and Delhey, 2007), with onion smudge being an important disease of white and yellow onions. Several *Colletotrichum* spp. have been reported as disease causing agents of onion smudge (Farr and Rossman, 2012). *C. gloeosporioides* and *C. coccodes* have straight conidia and infect primarily the leaves and necks of onion plants (Rodrguez-Salamanca et al., 2012), whereas, *C. circinans* causes primarily but not exclusively smudge symptoms on the bulbs and has falcate conidia. Pigmented onion scales contain protocatechuic acid which helps them fight onion smudge, a fungal disease caused by *Colletotrichum circinans* (Vitaglione et al., 2007).

Study of microorganism is generally facilitated if we are able to culture them under laboratory conditions. The composition of nutrient medium used to grow cells determine its rate of production, type of products produced and the yield of biomass and products. Elements like carbon, nitrogen, oxygen, sulphur and phosphorous must be provided in a suitable form

to produce the desirable biomass. (Chisti and Moo Young, 2003). Every laboratory experiment on plant pathogens start with culturing of casual fungi/bacteria. The composition and behaviour of plant pathogens is strongly dependent on their growth environment. So, we should give better attention to the culture media and culturing condition of microbes (Egli, 2015). Temperature is one of the external abiotic factors that has a significant impact on the growth of fungi. The majority of a fungus biological processes are influenced by temperature including growth, spore germination and reproduction (Lilly and Barnett, 1951). Temperature has been found to affect the *in vitro* mycelial growth rate of anthracnose pathogen (Nirenberg et al., 2002). Fungal virulence depends on the development of acervuli, differentiation of appressoria and growth of fungal hyphae. The growth rate of fungus depends upon temperature, humidity and surface nutrients. One of the vital components affecting fungal growth is temperature. At temperatures that are not feasible, growth rates decline or stop (Pasanen et al., 1991). Another important factor in the interactions between fungi and hosts is pH. The pH of a culture medium strongly influences fungal growth, sporulation and metabolite production (Child et al., 1973). The pH of the external environment can change the activity of enzymes like polygalacturonase (PG) and pectinlyase (PL) which changes the virulence level of disease (Mackenzie et al., 1993). Numerous physical factors like pH, temperature, light, aeration, etc., have a significant impact on growth of fungi. Fungi have a wider pH range than bacteria (between minimum and maximum values). While fungi generally prefer slightly acidic conditions for growth, the majority of microorganisms grows best in neutral (pH-7). However, some species prefer neutral to slightly alkaline conditions (Hogg, 2005). Fungi are able to grow and develop over a wider pH range than other microorganisms. Several species, including plant pathogens have the ability to actively modify the pH of their surroundings by releasing acids or alkalis (Landraud et al., 2013; Vylkova (2017). Keeping in view the importance of cultural parameters on growth and physiology of the microorganisms, present studies were conducted with an objective to find out best cultural parameters for the growth of *C. circinans.*

2. Materials and Methods

The study was conducted during October, 2021 to April, 2022 under laboratory conditions in the Department of Plant Pathology, College of Horticulture and Forestry, Neri (1189 m above mean sea level), Hamirpur, Himachal Pradesh, India.

2.1. Collection, isolation and identification of the pathogen

The diseased samples of onion showing typical symptoms of smudge were repeatedly washed in tap water. Thereafter, small pieces of the diseased tissues from infected portion of bulb were taken, along with some healthy tissue with the help of sterilized blade and surface sterilized with 1% sodium hypochlorite. After rinsing with sterilized distilled water, these tissues were placed on the sterilized blotting sheets to pat dry and were further placed on the potato dextrose agar (PDA) medium poured in sterilized Petri plates and slants with sterilized inoculating needle. The inoculated plates as well as slants were incubated at 28±2˚C in BOD incubator. After the initiation of the fungal growth in the inoculated plates/ slants, an agar bit from the periphery of actively growing mycelium was taken and placed on sterilized PDA slants to purify the culture. These purified cultures were then maintained in the refrigerator at 4–5˚C for use in further experiments. The pathogen was identified based on cultural and microscopic characters.

2.2. Effect of different nutrient media on the growth of C. circinans

Six different solid media *viz*., potato dextrose agar (PDA), Czapek's dox agar (CDA), Richard's agar (RA), malt extract agar (MEA), oat meal agar (OMA) and host extract agar (HEA) were evaluated for the growth of the test pathogen. A 5 mm bit of test fungus was taken with the help of sterilized cork borer from the pure culture plate and inoculated in the centre of the Petri plate already poured with respective medium and then incubated in BOD incubator at 28±2˚C. Each treatment was replicated thrice and data were recorded regularly at 24 h intervals upto five days (120 h) in terms of average diametric growth (mm) and cultural characteristics like colour of the mycelium, type of growth, growth pattern etc.

Growth rate (mm/h) of the fungus on each medium was further calculated as per the formula given below:

$$\text{Growth rate rg (mm h}^{-1}) = dgt_2\text{-}dgt_1/t_2\text{-}t_1$$

where: dgt_1 is the diametric mycelial growth (mm) at time t_1 and dgt_2 is the diametric mycelial growth (mm) at time t_2. Growth curves of the fungus between diametric growth and time of incubation on each test medium were also plotted. Further area under kinetic growth curve (rAUKC) values were calculated as per formula given by Bhogal et al. (2022) as below:

$$rAUKC=(dgt_2+dgt_1/2)\times t_2\text{-}t_1$$

The change in rAUKC i.e. ΔrAUKC values were further calculated for each time point by substracting the rAUKC value for each point from corresponding rAUKC value for next time points. The ΔrAUKC is an estimate of actual growth of the fungus, thus an estimate of actual growth rate of fungus. The ΔrAUKC values in each nutrient medium were plotted against various time intervals to see the trend of growth rate in different media with time.

Based on these studies, best nutrient medium was selected for further experiments.

2.3. Effect of different temperature regimes on the growth of C. circinans

To study the effect of different temperature regimes on the mycelial growth of the pathogen, Petri plates containing the best nutrient medium were inoculated with culture bit (5 mm dia.) of the test fungus and subjected to different

temperature regimes viz.,15, 20, 25, 30 and 35˚C in different BOD incubators upto 120 h. Each treatment was replicated five times and data were recorded regularly at 24 h intervals upto five days (120 h) in terms of average diametric growth (mm) and cultural characteristics as mentioned above. Based on these studies, best temperature was selected for further experiments. Growth rate and ΔrAUKC of the test pathogen at each temperature were plotted against time values were also plotted against time interval.

2.4. Effect of different pH levels on the growth of C. circinans

To study the effect of different pH levels on the mycelial growth of test pathogen, the best nutrient medium was adjusted to different pH levels viz., 5.0, 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0 with the help of 1N HCl and 1N NaOH and poured in Petri plates further for inoculation. Each petri plate was inoculated with a culture bit of 5 mm diameter. These Petri plates were then incubated at best temperature upto 120 h. Each treatment was replicated thrice and data were recorded regularly at 24 h intervals in terms of average diametric growth (mm) and cultural characteristics upto five days (120 h). Growth rate of the fungus at each pH level was further calculated as mentioned above. Growth rate as well as ΔrAUKC values of test fungus were also plotted against time.

2.5. Statistical analysis

Data recorded in three experiments were further subjected to statistical analysis for completely randomized design by using online software OPSTAT.

3. Results and Discussion

3.1. Collection, isolation and identification of the pathogen

The pathogen was isolated from a symptomatic onion bulb. The symptoms on infected bulb were recorded as small black spots on outer scales in circinate manner or concentric rings (Figure 1a). The fruiting bodies were formed on the stromata which developed beneath the cuticle of the host. One to several acervuli were formed on a single stroma. Microscopically, short and hyaline conidiophores were formed in a palisade layer and ruptured the cuticle of the host. The conidiophores were intermixed with brown coloured setae in between (Figure 1b). The mycelium of isolated pathogen was creamish white in colour having dense, cottony and supressed growth on PDA (Figure 1c). Microscopically, the mycelium was hyaline, septate and branched. The conidia were slightly curved or falcate and measured between 11.7–15.6 µm in length and 3.9–7.8 µm in width. However, in culture no acervuli were produced. Based on microscopic and cultural characters, the associated pathogen was identified as *Colletotrichum circinans* (Berk.) Voglino. The results are in conformity with the findings of Walker (1921), Kiehr et al. (2012) and Leylaie et al. (2014) who also reported *C. circinans with* similar characters to be associated with onion smudge.

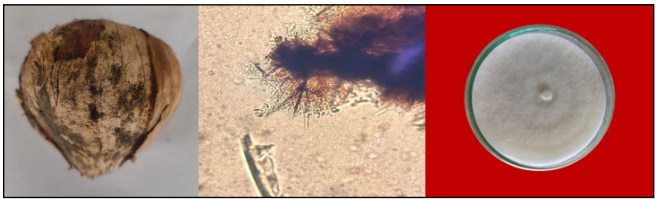

Figure 1: (a) An onion bulb exhibiting symptoms of smudge (b) Setae of *Colletotrichum circinans* from host tissue (c) Pure culture of *Colletotrichum circinans*

3.2. Effect of different nutrient media on mycelial growth of C. circinans

Data recorded in terms of average diametric growth (mm) after 120 h of incubation and growth rate of the test fungus, further calculated on the basis of mycelial growth of the fungus at 24 h interval upto 120 h on different test nutrient media have been presented in Table 1 and Figure 2. It is clear from the table that the fungus grew well on all the nutrient media under study. Significantly maximum diametric growth (90.00 mm) was recorded in PDA followed by the growth in HEA (83.10 mm), MEA (82.53 mm) and OMA (81.76 mm). However, mean minimum diametric growth was recorded in RA (46.96 mm) followed by CDA (59.00 mm) after 120 h of incubation. The growth curves obtained reflected linear to sigmoid growth in different phases of time. As far as growth rate of the fungus was concerned, irrespective of the time interval, mean growth rate of the test pathogen was significantly maximum in PDA (0.70 mm h^{-1}) which was statistically at par with HEA (0.65 mm h^{-1}) which further did not differ significantly from that in MEA (0.64 mm h^{-1}) and OMA (0.64 mm h^{-1}). However, minimum mean growth rate (0.35 mm h^{-1}) was recorded on Richard's agar followed significantly by Czapek's Dox agar (0.45 mm h^{-1}). Irrespective of the nutrient media under investigation, significantly maximum mean growth rate (0.86 mm h^{-1}) was recorded between 48–72 h of incubation while, the minimum growth rate (0.24 mm h^{-1}) was recorded between 0–24 h of incubation. Body of the table also reveals that average growth rate of the test fungus was maximum (1.15 mm h^{-1}) on MEA medium in between 48–72 h of incubation which was statistically at par with growth rate of test fungus on PDA between same duration (1.14 mm h^{-1}) while, minimum 0.02 (mm h^{-1}) growth rate was recorded on Richard's agar between 0–24 h of incubation which was statistically at par with that on OMA (0.10 mm h^{-1}) after same duration.

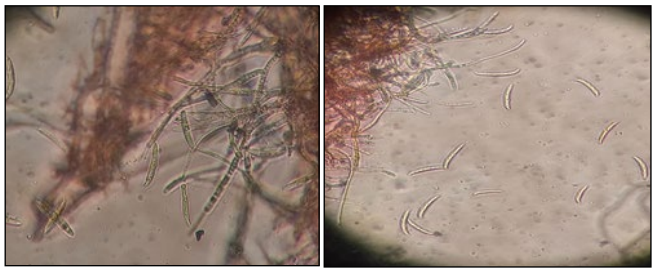

Figure 2: A micrograph of *Colletotrichum circinans* exhibiting mycelium and conidia

Table 1: Effect of different nutrient media on mycelial growth and growth rate of *Colletotrichum circinans*

Nutrient Medium	Average diametric growth (mm) after 120 h	Growth rate (mm h^{-1}) after time (h)					Overall mean
		0–24	24–48	48–72	72–96	96–120	
Potato dextrose agar	90.00	0.45	0.82	1.14	0.46	0.66	0.70
Czapek's dox agar	59.00	0.16	0.44	0.57	0.53	0.53	0.45
Malt extract agar	82.53	0.33	0.81	1.15	0.43	0.49	0.64
Richard's agar	46.96	0.02	0.17	0.51	0.57	0.48	0.35
Oat meal agar	81.76	0.10	0.96	0.89	0.71	0.52	0.64
Host extract agar	83.10	0.38	0.97	0.90	0.53	0.47	0.65
Overall mean		0.24	0.70	0.86	0.54	0.52	
				CD ($p \geq 0.05$)	SE(d)		
CD ($p \geq 0.05$)	1.31	Nutrient medium		0.06	0.03		
SE(d)	0.65	Time interval		0.05	0.02		
		Interaction		0.13	0.06		

The ΔrAUKC values plotted against time indicated a peak growth of PDA, HEA, MEA and OMA between 48–72 h of incubation while, in case of RA and CDA, the peak growth was recorded between 72–96 h of incubation after which, the growth declined (Figure 3).

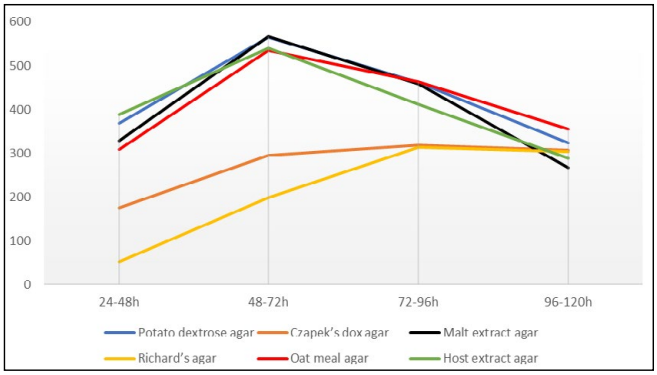

Figure 3: Difference in relative area under kinetic curve value plotted against time to depict the effect of nutrient media on growth of *Colletotrichum circinans*

Table 2: Effect of different nutrient media on cultural characters of Colletotrichum circinans

Nutrient medium	Colour of mycelium	Growth pattern	'Type of growth
Potato dextrose agar	Pure white	Ray	Cottony and fluffy
Czepek's dox agar	Transparent white	Ray	Sparse and thread like
Malt extract agar	Dull white	-	Cottony and fluffy
Richard's agar	Cremish white	Ray	Hairy growth
Oat meal agar	White	Ray	Strandy at center and cottony at margins
Host extract agar	Transparent to white	Ray	Strandy and sparsh at centre and cottony at margins

The data presented in Table 2 depict the Cultural characteristics of *C. circinans* recorded in each test nutrient medium revealed that colour of mycelium varied from transparent to pure white and dull white in different media under study. The growth pattern was observed to be ray in all the media under study except MEA. The type of growth in PDA and MEA was cottony and fluffy whereas, in CDA it was sparse and thread like. In case of RA hairy growth was observed, whereas, OMA and HEA growth was strandy at center and cottony at margins. Nutrients present in any nutrient medium directly or indirectly infuence the growth of any microorganism, due to their varied nutritional requirements for mycelial growth and sporulation. During present investigations, maximum mycelial growth was supported by PDA followed by HEA medium. Growth rate was also highest in PDA followed by HEA. The peak of growth rate was recorded between 48–72 h of incubation indicating that the fungus grows fastest in this duration and after that the nutrients start exhausting leading to a gradual decline in the growth rate. The colour of mycelium varied from pure white to cream. The growth pattern was generally ray pattern. The present findings were in accordance with Walker (1921) who concluded that potato dextrose agar was best media. The results are further supported by the findings of Akhtar and Singh (2007) who reported that in *C. capsici* highest mycelial growth was observed in PDA. The findings of Rajesha and Mantur (2014) and Lokare et al. (2021) also strengthen our

results who reported PDA to be the best supportive medium for the growth of different *Colletotrichum* spp. However, fastest growth rate of the test fungus on PDA can also be attributed to the availability of more nutrients in this medium ultimately leading to faster metabolism and growth. Based on these studies PDA was selected as the best medium and used in subsequent experiments.

3.3. Effect of different temperature regimes on mycelial growth of the C. circinans

Data presented in Table 3 clearly depict that significantly maximum average diametric growth (90.00 mm) was recorded at 30°C after 120 h of incubation which was statistically at par with that at 25°C (89.66 mm) after same period of incubation. However, significantly minimum average diametric growth (5.00 mm) was recorded at 15°C after all the durations of incubation which was statistically at par with the growth at 20°C (6.56 mm h^{-1}) and 35°C (5.05 mm) after 24 h duration of incubation. It was interesting to note that the fungus did not grow at all at 15°C as the same diametric growth was recorded up to 120 h of incubation. Growth rate of the test fungus at different temperatures under study was recorded to be maximum (0.71 mm h^{-1}) at 30°C which was statistically at par with that recorded at 25°C (0.70 mm h^{-1}) followed significantly by growth rate (0.44 mm h^{-1}) at 35°C. However, minimum mean growth rate (0.30 mm h^{-1}) of test fungus was recorded at 20°C. Irrespective of different temperature regimes, maximum mean growth rate (0.77 mm h^{-1}) was recorded between 24–48 h of incubation which was statistically at par with growth rate (0.71 mm h^{-1}) between 96–120 h of incubation. However, significantly minimum mean growth rate (0.08 mm h^{-1}) of the test fungus was recorded between 0–24 h of incubation. As fungus totally failed to grow at 15°C, thus growth rate was not calculated at this temperature. The body of the table reveals that growth rate of the test fungus was maximum (0.94 mm h^{-1}) at 24–48 h of incubation which was statistically at par with growth rate recorded at 30°C (0.93 mm h^{-1}) between 24–48 h and 25°C as well as 30°C (0.92 mm h^{-1}) between 96–120 h of incubation. However, significantly minimum growth rate (0.01 mm h^{-1}) of the test fungus was recorded at 35°C between 0–24 h of incubation, which was statistically at par with growth rate recorded at rest three temperatures between same duration of incubation (Figure 4).

Table 3: Effect of different temperature regimes on cultural characters of *Colletotrichum circinans*

Temperature (°C)	Colour of mycelium	Growth pattern	Type of growth
15	-	-	-
20	Transparent at center and white at margin	Ring	Fibrous and cottony
25	Transparent at center and white at margins	Ring as well as ray	Fibrous and cottony
30	White	Ring	Standy and cottony
35	Transparent and white alternate rings	Typical ring pattern	Cottony

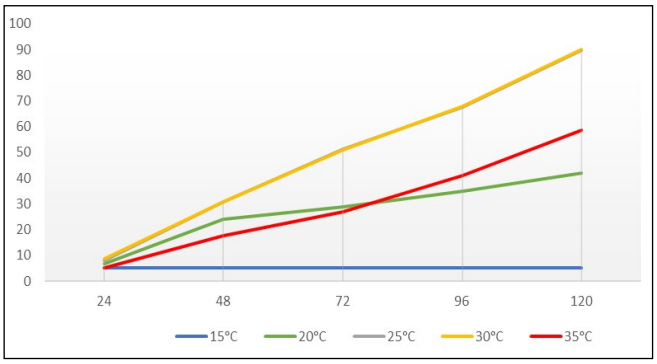

Figure 4: Growth curves of *Colletotrichum circinans* under the influence of different temperature regimes

The ΔrAUKC values plotted against time indicated a peak in growth rate at temperature 20°C, 25°C and 30°C between 48–72 h of incubation. At temperature 35°C relative growth reached its peak between 96–120 h (Figure 5).

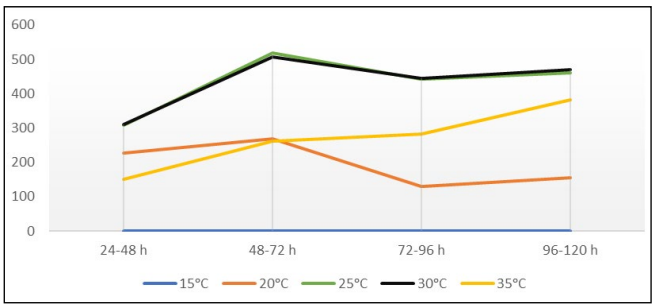

Figure 5: Difference in relative area under kinetic curve values plotted against time to depict the effect of different temperatures on growth of *Colletotrichum circinans*

The data presented in Table 2 depict the colour of mycelium varied from transparent at center and white at margins at 20 and 25°C.Whereas, it was completely white at 30°C and alternate ring of transparent and white at 35°C. The growth pattern was observed to be ring at temperature 20, 30 and 35°C while, at 25°C ring as well as ray pattern of growth was observed. As far as type of growth was concerned, it varied from fibrous and cottony at 20 and 25°C to strandy and cottony at 30°C. Cottony growth was however observed at 35°C.

Temperature of incubation also affected the mycelial growth, rate of growth and growth pattern of *C. circinans.* According to our experiments, optimum temperature for growth of *C. circinans* recorded was 25 to 30°C. But, the fungus grew well at temperature ranging from 20 to 30°C. Our findings are supported by the results of Hubballi et al., 2011 and

Lehrwan et al., 2018 who also reported 25–30°C to be the best temperature for the growth of *C. gloeosporioides.*

3.4. Effect of different pH levels on mycelial growth of the C. circinans

It is clear from the table 4 that the maximum mean diametric growth (90.00 mm) was recorded at pH 7.0 after 120 h of incubation which was statistically at par with growth of test fungus recorded at pH 7.5 (88.66 mm) after same duration. However, minimum average diametric growth (6.10 mm) was recorded at pH 5.5 after 24 h of incubation and interestingly, it was statistically at par with the diametric growth of test fungus at all test pH levels after 24 h of incubation except that at pH 6.0 (8.73 mm).

Table 4: Effect of different pH levels on cultural characters of Colletotrichum circinans

pH	Colour of mycelium	Growth pattern	Type of growth
5.0	White	-	Thick fibrous to cottony
5.5	Cottony white	Ray	Fine fibrous
6.0	White	Ring	Cotton candy growth
6.5	White	Ring	Cotton candy
7.0	Transparent at centre and white at margins	Ray	Cottony
7.5	Transparent at center and white at margins	-	Fibrous cottony
8.0	White	-	Fibrous cottony

It is clear from the table that irrespective of the time interval, mean maximum mean growth rate (0.70 mm h^{-1}) of the test pathogen was recorded at pH 7.0, which was statistically at par with that at pH 7.5 (0.69 mm h^{-1}) and 8.0 (0.66 mm h^{-1}) while, minimum mean growth rate was recorded at pH 5.5 (0.38 mm h^{-1}) which was statistically at par with that at pH 5.0 (0.41 mm h^{-1}), 6.0 (0.43 mm h^{-1}) and 6.5 (0.42 mm h^{-1}). Irrespective of different pH levels under study, significantly maximum mean growth rate (0.77 mm h^{-1}) was recorded between 96–120 h of incubation while, significantly minimum mean growth rate (0.12 mm h^{-1}) was recorded between 0–24 h of incubation. Body of the table reveals that the maximum growth rate (1.24 mm h^{-1}) was recorded at pH 8.0 in between 96–120 h of incubation which was statistically at par with growth rate between same duration of incubation at pH 7.5 (1.21 mm h^{-1}) and 7.0 (1.10 mm h^{-1}). However, minimum growth rate (0.04 mm h^{-1}) was recorded between 0–24 h of incubation at pH 5.5 which was statistically at par with growth rate of the test fungus at rest all pH levels between same duration of incubation (Figure 6).

ΔrAUKC values plotted against time clearly depict that ΔrAUKC

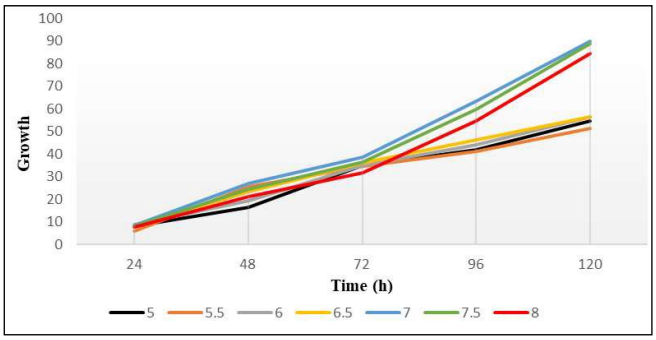

Figure 6: Growth curves of *Colletotrichum circinans* under the influence of different pH levels

values at pH 7.0, 7.5 and 8.0 obtained a peak between 96–120 h of incubation. Whereas, at pH 5.0, 5.5, 6.0 and 6.5 a peak was obtained at 48–72 h of incubation and thereafter, the growth declined sharply (Figure 7).

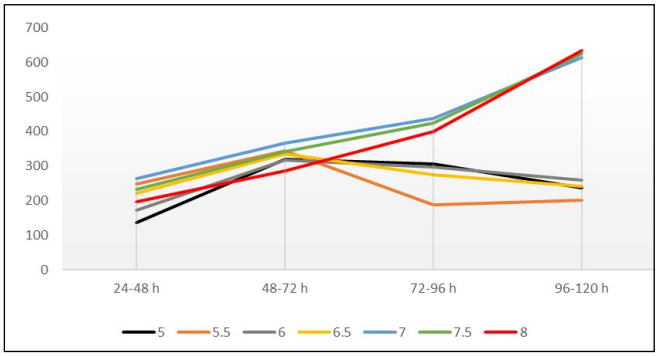

Figure 7: Difference in relative area under kinetic curve values plotted against time to depict the effect of the different pH levels on growth of *Colletotrichum circinans*

The colour of mycelium was white at different pH levels except at pH 7.0 and 7.5 where, the colour was transparent at center and white at margins. The growth pattern was observed to be ring and ray at pH 6.5 and 7.0 respectively, while, no specific pattern was recorded at rest of the pH under study. Type of growth was recorded to be cottony at pH 7.0 while, it was cotton candy at pH 6.0 and 6.5. At pH 5.0 and 5.5 the growth was thick fibrous to cottony and fine fibrous, respectively. However, fibrous cottony growth was observed at pH 7.5 and 8.0. Studies on effect of pH levels on mycelial growth of *C. circinans* revealed that pH 7.0 supported the best growth of fungus with highest rate of growth followed by pH 7.5 and 8.0. The fungus grew well at all the pH levels under study, with optimum range being pH 7.0–8.0. The present findings are in agreement with the findings of Akhtar et al., 2018 who reported pH 7.0 to be the best pH for the growth of *C. capsici*. However, the results are more or less in conformity with the findings of Hubbali et al., 2011 who reported pH 6.5 and 7.0 to be the best for the growth of *C. gloeosporioides*.

4. Conclusion

The onion smudge occurs in mild to severe form in non-

pigmented onion bulbs while, pigmented bulbs are resistant to disease. Symptoms appeared to be small round dark spots scattered over the surface of bulb in concentric rings of diameter 1–2 cm. The fungus grew well on potato dextrose agar having pH 7.0 at 30°C.

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