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Cultural analysis and growth kinetics of *Cephaleuros virescens* Kunze inciting red rust in litchi

Garima, Kumud Jarial, RS Jarial and Aakash

Abstract
Different nutrient media, temperature regimes, pH levels and photoperiods were evaluated to determine the best physiological conditions for the growth of *Cephaleuros virescens*. Among the six different nutrient media tested, agarized host leaf extract was found to be the best medium for maximum mycelial growth (51.11 mm) and growth rate (0.95 mm) with initially white to dull white mycelial growth that later turned green with time. Out of seven different temperature regimes tested, 25 °C was found to be the best temperature for mycelial growth (52.44 mm) with maximum growth rate (0.93 mm). Among the seven pH levels tested, pH 8.5 was found to be the best for promoting maximum mycelial growth (53.39 mm) and growth rate (0.88 mm). Among the photoperiods tested, 12h light conditions proved to be the best photoperiod in terms of maximum growth (65.35 mm) and growth rate (0.93 mm).

Keywords: *Cephaleuros virescens*, Cultural characters, growth rate, red rust

Introduction
Litchi chinensis is the most important subtropical evergreen tree in the family *Sapindaceae* and subfamily *Nephelaceae* (Mitra, 2002) [11]. Litchi is thought to have originated in China, and by the end of the 17th century, it had spread to Burma and India. It is a high-priced fruit in India also known as the "Queen of Fruits." Diseases are one of the constraints in the fruit production of litchi as they directly or indirectly reduce the yield and quality of fruit (Anal *et al.*, 2017) [2]. Algal leaf spot or red rust disease is the one which is caused by alga *Cephaleuros virescens*. The infections incited by *Cephaleuros* species are also referred to as algal rust or red rust and are often confused with the rust disease caused by fungi (Muthukumar *et al*., 2014) [12]. The genus *Cephaleuros* belongs to the division of aquatic green algae *Chlorophyta*, class *Ulvophyceae*, order *Trentepohliales* and family *Trentepohliaceae*. They need free water to germinate (Sunpapao *et al*., 2016) [15]. Algal leaf spot is considered a threat during nursery crop production because it can cause primary (direct) and can also lead to secondary (indirect) infection (Browne *et al.*, 2019) [4]. If the disease is spotted on high-value fruits or plant leaves, it can have a significant impact on the economic value of the plant hosts. The damage caused by *Cephaleuros* species results from the reduction in the leaf photosynthetic area, which may represent economic loss, especially in times of high temperature and humidity (Pereira *et al.*, 2020) [13].

Materials and methods
The investigation was conducted in the Research Laboratory, Department of Plant Pathology, College of Horticulture and Forestry, Neri, Hamirpur, Himachal Pradesh, India.

Isolation, identification and pathogenicity of *Cephaleuros virescens*
The leaves infected by red rust disease were collected and brought to the laboratory for isolation and purification of the associated pathogen. The samples were thoroughly washed repeatedly under tap water. Small pieces of the diseased tissues were taken from the lesions on infected leaves along with some healthy portion with the help of sterilized blade and surface sterilized with 0.1 per cent mercuric chloride for 10-15 seconds. Thereafter, these tissues were rinsed with sterilized distilled water thrice, kept on sterilized blotting paper to pat dry and inoculated on Bold's basal medium (BBM) poured in sterilized Petri plates and slants with sterilized inoculating needle. The inoculated plates and slants were incubated at room temperature under 12h light /12h dark conditions. After the initiation of the growth of algal mycelium, a cultured bit was transferred to sterilized BBM slants to purify the culture. These purified cultures were then maintained in the refrigerator for use in further experiments.

The pathogen was identified on the basis of cultural and microscopic characters of the alga.

To prove pathogenicity, fully grown algal culture on Bold's basal medium was scrapped and ground in mixer-grinder so as to obtain very small fragments of the alga in sterile water. This suspension was sprayed on the healthy litchi seedlings. The leaves were first wounded with pin pricks and then inoculated with algal suspension. Wounded leaves sprayed with sterile water alone served as control. These seedlings were then covered with polythene bags internally sprayed with sterile water so as to maintain appropriate relative humidity. Observations were recorded in terms of incubation period. In another pathogenicity experiment, the alga was cultured on Bold's Basal medium for upto one month till the formation of gametangia like bodies in the culture. Thereafter, the mycelium of the alga along with gametangia like bodies was scrapped from the whole Petri plate and put in sterilized distilled water (100 ml) in Erlenmeyer flask (150 ml capacity). This mixture was shaken vigorously. This suspension was then sprayed on the healthy leaves of litchi seedlings already pin pricked. These plants were then covered with transparent polythene bags internally sprayed with sterilized distilled water to maintain the appropriate relative humidity (RH). The polythene bags were sprayed regularly with sterilized distilled water to maintain the RH till the appearance of symptoms. Healthy leaves of seedlings pin pricked and sprayed with sterilized distilled water alone served as control. Data were recorded in terms of incubation period (days).

Effect of different nutrient media on the growth of *Cephaleuros virescens*

Six different media *viz.,* potato dextrose agar (PDA), potato sucrose agar (PSA), Bold's basal medium (BBM), agarized host leaf extract (HLE), Trebouxia medium and Bristol medium were evaluated for the growth of the *C. virescens*. A 5 mm bit of test alga was taken with the help of sterilized cork borer from the pure culture plate and inoculated in the centre of Petri plate poured with respective medium and then incubated at 28±1°C under 12h light and dark conditions. Each treatment was replicated three times and data were recorded regularly at 24 h intervals up to four days in terms of average diametric growth (mm) and cultural characteristics like type of growth, growth pattern, colour of the mycelium etc. Additionally, observations were also recorded in terms of formation of gametangia like bodies. Based on the diametric growth of alga in different media at particular point of time, growth curves were plotted by taking mycelial growth verses time on Y and X axis, respectively. Growth rate (mm/h) of the alga on each medium was further calculated as per the formula given below:

Growth rate r_g (mm/h) = dgt_2-dgt_1/t_2-t_1

Where

$dgt_1$and dgt_2 are the values of diametric growth at time t_1 and t_2, respectively.

Additionally, to visualize small changes in the growth rates during the growth curve and to determine the time point after which growth rate changed significantly, a calculation model based on area under kinetic curve (AUKC) was developed. With this model, the relative AUKC (rAUKC) at each interval was estimated by using following formula:

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

The changes in rAUKC (ΔrAUKC) were calculated for each time point by subtracting the rAUKC for each time point from the corresponding rAUKC of the previous time point. The ΔrAUKC is an estimate of changes in the slope of the growth curve and thus an estimate of the growth rate of alga. When the ΔrAUKC value increases linearly over time, mycelial growth increases at a constant rate, and when it decreases or goes to zero over time, the growth rate decreases or goes to zero. As a result, increasing ΔrAUKC values corresponded to rapid growth rates. The ΔrAUKC values were used to distinguish different phases of algal growth and the time spent in each phase. To see the trend of growth rate in different media with time, rAUKC values in each nutrient media were plotted against various time intervals. Based on these findings, the best nutrient medium was chosen for further experiments.

Effect of different temperature regimes on the growth of the test pathogen

To evaluate the effect of different temperature regimes on the mycelial growth of the alga, Petri plates containing the best nutrient medium were inoculated with 5 mm culture bit of the test pathogen and subjected to different temperature regimes *viz.,* 17, 20, 22, 25, 28, 30 and 32°C under artificial conditions with 12 h photoperiod upto 96 h. Each treatment was replicated three times and data were recorded regularly at 24 h intervals in terms of the average diametric growth (mm) and cultural characteristics as previously mentioned. Growth rate, rAUKC and ΔrAUKC values of the alga at each temperature were further calculated as mentioned earlier. Based on these studies, best temperature was selected for further experiments.

Effect of different pH levels on the growth of the *C. virescens*

To study the effect of different pH levels on the mycelial growth of *C. virescens*, the best nutrient medium was adjusted to different pH levels *viz.,* 6.0, 6.5, 7.0, 7.5, 8.0, 8.5 and 9.0 with the help of 1 N HCl and 1 N NaOH and poured in Petri plates and further inoculated with a culture bit of 5 mm diameter. These Petri plates were then incubated at best temperature up to 96 h with 12 h photoperiod to record the data. Each treatment was replicated thrice and data were recorded regularly at 24 h intervals in terms of average diametric growth (mm) and cultural characteristics up to 96 h. Growth rate, rAUKC and ΔrAUKC values of the alga at each pH level were further calculated.

Effect of different photoperiods on the growth of the test pathogen

To study the effect of different photoperiods on the mycelial growth of the test pathogen, the best nutrient medium was adjusted at best pH and poured in Petri plates and inoculated with culture bit of 5 mm diameter. The inoculated plates were incubated at best temperature under different photoperiods *viz.,* 12h/12h, 9h/15h, 6h/18h, 3h/21h and 0h/24h light/dark conditions, respectively. Each treatment was replicated four times and data were recorded regularly at 24 h intervals up to 96 h in terms of average diametric growth (mm) and cultural characteristics. Growth rate, rAUKC and ΔrAUKC values of the alga at each treatment were further calculated.

Statistical Analysis

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

Results and discussion

Isolation, identification and pathogenicity of the associated pathogen

The symptoms on leaves (Fig. 1) were recorded as orange, rust coloured, velvety spots which later led to curling of leaves in the presence of mites. The cross sections of infected leaves revealed aerial sporangiophores and mycelium which was orangish red in colour. The pathogen was isolated and purified on Bold's basal medium (BBM) and maintained in BBM slants at 4-5 ℃ in the refrigerator. The mycelium was initially whitish in colour which later turned green having root like growth and ray pattern of growth was observed in the Petri plate (Fig. 2). On the basis of morphological, cultural and microscopic characters, the pathogen was identified as *Cephaleuros virescens* Kunze. Microscopically, the mycelium was greyish to light brown, septate and vesiculate having varying diameter at points. At later stages, sporangia / gametangia like bodies were produced in the mycelium (Fig. 3) which turned the mycelium to be velvety in culture.

In the pathogenicity study, at first, the plants did not exhibit many symptom of the disease except for one or two leaves on which, orange circular spots were observed on upper leaf surface (Fig. 4) after 24 days. The algal sporangiophores and sporangia were observed under microscope when these spots were cut into transverse sections (Fig. 4). In another experiment on pathogenicity, orangish spots were formed on abaxial side of leaves after 2 weeks which looked like faded spots on the corresponding adaxial surface. The sporulating mycelium was observed after 10 days of appearance of symptoms. At later stages *i.e.* almost after a month, the spots turned rusty. On abaxial surface of leaves, sunken rusty spots were observed and on the opposite side of leaf, elevated orange spots could be seen at the corresponding points. These symptoms started appearing after two weeks of inoculation and could be observed appearing up to three months of inoculation on different leaves. According to microscopic observation, the pathogen was confirmed to be *C. virescens*. Thus, the symptoms firstly appeared as orangish spots on abaxial surface leading to fading of green colour of leaves on corresponding adaxial surface, which later turned as superficial green coloured mycelium ultimately leading to rusty orange spots on abaxial as well as adaxial portion of leaves (Fig. 5).

Although, most of the researchers have failed to obtain typical symptoms of red rust in artificially inoculated plants (Holcomb *et al*., 1998; Brooks, 2004 and Kumar *et al*., 2019) [7, 3] but, our results are supported by Sanahuja *et al.* (2018) [14] who revealed that symptoms of red rust disease were observed after 8 weeks of first inoculation on *Neoregelia bromeliads* caused by *Cephaleuros parasiticus*.

Effect of different nutrient media

Data recorded in terms of average diametric growth (mm) after 96 h of incubation and growth rate of the test alga, further calculated on the basis of mycelial growth of the alga at 24 h interval upto 96 h on different test nutrient media have been presented in Table 1. The alga grew well on all the nutrient media under study. Irrespective of the time of observation, significantly maximum mean diametric growth was recorded when *C. virescens* was grown on agarized HLE (51.11 mm) followed by that on PDA (35.28 mm) and PSA (21.11 mm). The minimum mean diametric growth was recorded on Bristol medium (12.28 mm) followed significantly by Trebouxia medium (14.36 mm) and BBM (15.43 mm). Irrespective of the nutrient media under study, significantly maximum mean diametric growth (40.89 mm) of the alga was recorded after 96 h of incubation while, significantly minimum mean diametric growth (9.33 mm) was recorded after 24 h of incubation. A significant increase in the average diametric growth of the mycelium was recorded after each 24 h interval of incubation.

The body of the table reveals that the maximum diametric growth (95.89 mm) was recorded after 96 h of incubation on agarized HLE followed significantly by growth in the same medium (69.00 mm) after 72 h of incubation. However, minimum diametric growth was recorded on Bristol medium (5.44 mm) after 24 h of incubation which was statistically at par with BBM (5.67 mm) after same duration which further did not differ significantly from the growth in Trebouxia medium (6.67 mm) after same duration. An intermediate diametric growth was recorded in rest of the growth media after different durations of incubation.

The Fig. 6 clearly depicts that the growth of test alga was fastest on agarized HLE medium. A sigmoid curve was obtained with the growth of alga on HLE medium which shows that the pathogen grew well at optimum speed on this medium. Moreover, Bristol medium also showed sigmoid curve but the growth was slow in comparison to agarized HLE as stationary phase was observed very soon. Bold's basal medium was found to be favourable for growth of *C. virescens* but again the growth was slow and at later stages growth was not found to increase. PDA was found to be more appropriate medium in comparison to PSA.

Effect of different nutrient media on growth rate of *Cephaleuros virescens*

A review of the data in Table 2 reveals that, regardless of time interval, the maximum mean growth rate (0.95 mm) was recorded when *C. virescens* was inoculated on agarized HLE, followed by PDA (0.54 mm/h) and BBM (0.24 mm/h). However, the minimum mean growth rate (0.15 mm/h) was recorded on Trebouxia medium which was statistically at par to that on Bristol medium (0.16 mm/h), followed by PSA (0.21 mm/h). The maximum mean growth rate (0.53 mm/h) was recorded between 48-72 h of incubation, while the minimum mean growth rate (0.18 mm/h) was recorded between 0-24 h of incubation, regardless of the nutrient media under investigation.

The body of the table reveals that the average growth rate of the test alga was maximum (1.68 mm/h) on agarized HLE between 48-72 h of incubation and minimum (0.02 mm/h) on Bristol medium between 0-24 h of incubation, which was statistically at par to BBM (0.03 mm/h) and Trebouxia medium (0.07 mm/h) between same duration of incubation.

The Fig. 7 shows difference in relative area under kinetic curve (ΔrAUKC) values plotted against time which depict the peak growth of test alga between different duration of incubation on different nutrient media. It is clear from the figure that the peak growth of HLE, BBM and Bristol was between 72-96 h of incubation and actually the alga was still in the process of growth after 96 h indicating these media to

be supportive for the alga. In rest of the media, the algal growth declined after certain time of incubation (after 48 h in PSA and after 72 h in PDA and Trebouxia) indicating that these media were less supportive for the algal growth.

The colour of mycelium (Table 3) at initial stages varied from transparent white to dull white which later turned green due to pigmentation in most of the media under investigation except in Bristol medium where no pigmentation was observed and Trebouxia medium where only green threads were observed. As far as the growth pattern was concerned, it varied from ring to ray pattern in most of the media (PLATE 6). The colour of mycelium was observed to be white to dull white in PDA, PSA and Trebouxia medium while, it was dark to light transparent in BBM, Bristol and Agarized HLE media which later turned green with progression of time.

The type of growth in PDA and PSA was cottony and fluffy while, it was root like and velvety in BBM. On the other hand, suppressed growth was visualised in Trebouxia medium and Agarized HLE, while in Bristol medium, it was hairy/ thread like growth. Gametangia like bodies were also visualized microscopically in PDA, BBM and HLE media (Fig. 5).

The nutrients in the nutrient medium either directly or indirectly influenced alga growth. During present investigations, maximum mycelial growth was supported by Agarized HLE followed by PDA medium. Agarized HLE had the fastest growth rate, followed by PDA. The present findings were in accordance with Kumar *et al*. (2019), who concluded that algal thalli were successfully isolated and cultured on BBM and PDA, but no sporangiophores or sporangia developed in culture. However, the findings contradict those of Vasconcelos *et al*. (2018) [17], who recorded the highest mycelial growth average in Petri dishes when the algae were grown in Trebouxia and BBM media (8.2 to 8.0 cm). However, the mycelial growth in Petri dishes upto day 4 better fitted a linear model for all culture media which support our findings. Furthermore, the results are also supported by Wolf (1930) [18] who observed that *C. virescens* cultured on agar medium containing dextrose and mineral salts showed cushion-like filament mass. According to Bunjongsiri and Sunpapao (2018) [15], five *Cephaleuros* species formed reproductive structures such as gametangia-like bodies and sporangia, but no zoospores or gametes were observed on agar media, confirming the current findings. However, because there are no reports in the literature regarding the growth rate of the alga on different nutrient media, these findings cannot be compared to any available data.

Best growth in HLE can be explained by the fact that plant juices and/or extracts are well suited as culture media for microbial growth, as they contain all the necessary nutrients as well as growth factors such as vitamins, minerals and amino acids. Also, according to findings of Bunjongsiri and Sunpapao (2018) [15], Bold's basal medium amended with indole-3-acetic acid (IAA) was recorded to be the most suitable nutrient medium for growth of *Cephaleuros* species when compared with plain BBM. Auxins are present in leaves, growing shoots and buds (Costa *et al.*, 2017) [6]. Therefore, our findings can further be explained by the fact that litchi leaf extract might be containing auxin or same growth promoting chemicals which could support the growth of test alga and thus, proved as best medium. Based on these studies, agarized HLE was selected as best medium and used in subsequent experiments.

Effect of different temperature regimes

The data in Table 4 clearly show that the maximum mean diametric growth (52.44 mm) was recorded at 25 °C, followed by growth at 28 °C (51.75 mm) and 22 °C (47.69 mm), which was statistically at par to that at 30 °C (47.47 mm). However, the mean minimum diametric growth (17.17 mm) was measured at 17 °C, followed by 20 (38.50 mm) and 32 °C (42.17 mm). Regardless of temperature regime, the maximum mean diametric growth (79.31 mm) was recorded after 96 hours of incubation, while the minimum mean diametric growth (8.54 mm) was recorded after 24 hours. After each 24 hour incubation period, diametric growth increased significantly.

The interaction of different temperature regimes and time of incubation reveals that maximum average diametric growth (94.78 mm) was recorded at 28 ℃ after 96 h of incubation which was statistically at par with 25 ℃ (94.56 mm) after same period of incubation. However, significantly minimum average diametric growth (5.00 mm) was recorded at 17 ℃ after 24 h of incubation.

The growth curve of all the temperatures under investigation was recorded to be sigmoid but rate of growth was high when incubated at temperatures 28 and 25 ℃ followed by 22 ℃. However, at 17 ℃, slow growth as compared to other treatments was observed (Fig. 9).

Effect of different temperature regimes on growth rate of *Cephaleuros virescens*

Growth rate of the test alga at different temperatures under study was also calculated and have been presented in Table 5.

The table clearly shows that, regardless of time interval, the mean growth rate of the test pathogen was maximum and equal (0.93 mm/h) at both 25 and 28 °C, followed by that at 22 (0.87 mm/h) and 30 °C (0.85 mm/h). However, the alga's minimum mean growth rate (0.36 mm/h) was recorded at 17 °C, which was significantly followed by 20 °C (0.73 mm/h) and 32 °C (0.79 mm/h). Irrespective of the temperature regimes, the maximum mean growth rate (1.19 mm/h) was recorded between 48-72 h of incubation, followed by 72-96 h (1.01 mm/h) and 24-48 h (0.79 mm/h), while the minimum mean growth rate (0.15 mm/h) was recorded between 0-24 h of incubation.

The body of the table indicates that the growth rate of test alga was maximum (1.45 mm/h) at 30 °C between 48-72 h of incubation, followed significantly by that at 32 °C (1.41 mm/h) between same period of incubation, which did not differ significantly from the growth rate at 28 °C (1.38 mm/h) between same period of incubation. However, no growth (0.00 mm/h) of the test alga was observed at 17°C between 0-24 h of incubation, followed by growth rates (0.07 mm/h) at 20 and 32 °C during the same time period. The remaining treatments showed an intermediate range of growth rates.

Difference in relative area under kinetic curve values plotted against time (Fig. 10) clearly depict that at six out of seven temperatures under study, relative growth continuously increased and reached its peak between 72-96 h of incubation except at 17 ℃ where the growth at initial stages declined between 24-48 h of incubation and then again increased continuously reaching its peak of relative growth between 72-96 h of incubation. A sigmoid curve was obtained at all temperatures except at 17 ℃.

As depicted in Table 6, the colour of mycelium varied from transparent white to whitish grey which later turned out to be light green. The growth pattern was observed to be ring pattern except at temperature 20 ℃ which exhibited ring as well as ray pattern. As far as type of growth was concerned, it varied from filamentous, cottony and fluffy to suppressed at different temperatures under study (Fig. 11).

According to our experiments, best temperature for growth of *C. virescens* recorded was 25 ℃ followed by that at 28 ℃. But the alga grew well at temperature ranging from 20 to 30 ℃. The growth rate at 25 and 28 ℃ was however same. Our findings are in accordance with Suto and Ohtani (2011) [16] who reported that colonies of *Cephaleuros* species were formed at temperatures ranging from 5 to 30 °C or upto 35 °C with an optimum at 25 °C, but thalli also grew well at 20 °C and 30 °C. The results are further strengthened by Lavens and Sorgeloos (1996) [9] who reported that most cultured algae tolerate temperatures between 16-27 ℃ and temperatures below 16 ℃ slow down the growth whereas, those higher than 35 ℃ are lethal for many species. According to Probert and Klaas (1999), the algal cultures should be maintained at temperatures as close as possible to the temperature at which it was collected. *C. virescens* causing red rust in litchi is more present during the rainy season in subtropical area of Himachal Pradesh and the prevalent temperature in this zone during this period ranges between 20-30 ℃. In our findings also, 25-28 ℃ was found to be optimum for cultural growth of alga.

Effect of different pH levels on mycelial growth of the *Cephaleuros virescens*

The data presented in the Table 7 clearly indicate that irrespective of duration of incubation, maximum mean diametric growth (53.39 mm) was recorded at pH 8.5 which was statistically at par with the growth at pH 8.0 (53.38 mm) and pH 7.5 (53.28 mm) while, significantly minimum mean diametric growth (47.12 mm) was recorded at pH 6.0 followed by pH 6.5 (49.19 mm). Irrespective of different pH under study, mean maximum diametric growth (88.18 mm) was recorded after 96 h of incubation while, mean minimum diametric growth (10.27 mm) was recorded after 24 h of incubation. A significant increase in the diametric growth of the alga was recorded at each 24 h interval.

The body of the table reveals that the maximum mean diametric growth (89.56 mm) was recorded at pH 7.5 after 96 h of incubation which was statistically at par with the growth of alga at pH 8.0 (89.45 mm) and 9.0 (89.26 mm) after same duration. However, minimum average diametric growth (5.83 mm) was recorded at pH 6.0 after 24 h of incubation.

The area under growth curves (Fig. 12) influenced by different pH values clearly depict that the alga grew well at all the pH levels under study. At pH range between 7-9, sigmoid curves were obtained proving it to be optimum range for the growth of alga. However, straight lines were observed at pH 6 and 6.5.

Effect of different pH levels on growth rate of *Cephaleuros virescens*

The data presented in the Table 8 clearly indicate that irrespective of the time interval, maximum and equal mean growth rate (0.88 mm/h) of the test alga was recorded at pH 7.5, 8.0 and 9.0 which was statistically at par with that at pH 8.5 (0.87 mm/h) while, minimum mean growth rate was recorded at pH 6.5 (0.84 mm/h) which was statistically at par with pH 6.0 (0.85 mm/h) and pH 7.0 (0.85 mm/h). Irrespective of different pH levels under study, significantly maximum mean growth rate (1.30 mm/h) was recorded between 48-72 h of incubation while, significantly minimum mean growth rate (0.22 mm/h) was recorded between 0-24 h of incubation.

Body of the table reveals that the maximum growth rate (1.39 mm/h) was recorded at pH 9.0 between 48-72 h of incubation which was statistically at par with growth rate (1.38 mm/h) between same duration of incubation at pH 8.0 and pH 8.5 (1.36 mm/h). However, the minimum growth rate (0.03 mm/h) of the test alga was recorded between 0-24 h of incubation at pH 6.0.

It is clear from Fig. 13 that difference in relative area under kinetic curve values obtained a peak between 48-72 h of incubation and after that the values declined sharply between 72-96 h. This indicated that the difference in growth (growth rate) of the alga was quite high between 48-72 h of incubation at all pH levels and declined sharply after that.

As far as mycelial characters of the alga at different pH levels were concerned (Table 9), the colour of mycelium varied from dull white to greenish grey except at pH 8.5 and 9.0 which exhibited cottony white and greyish green colour, respectively. The growth pattern was observed to be ring except at pH 8.5 where the mycelium was evenly spread (Fig. 14). Type of growth was recorded to be suppressed to cottony at various pH levels under study.

Investigations on the effect of pH levels on mycelial growth of *C. virescens* revealed that pH 8.5 supported the best growth of alga with highest rate of growth followed by pH 8.0 and 7.5. The alga grew well at all the pH levels tested, with optimum range being pH 7-9. These findings are in accordance with Lavens and Sorgeloos (1996) [9] who reported that the pH range for most cultured algal species is between 7 and 9, with 8.2-8.7 being the optimum range. Furthermore, the results are also supported by the findings of Suto and Ohtani (2011) [16] who revealed that colonies of alga were formed on agar media with a pH ranging from 3.1 to 8.7, with an optimum at pH 5.5–7.6. Based on these studies, pH 8.5 was selected as the best pH level and was used in subsequent experiments.

Effect of different photoperiods on mycelial growth of *Cephaleuros virescens*

It is evident from the Table 10 that irrespective of the time interval, maximum significant mean diametric growth (65.35 mm) was recorded at 12 h/ 12 h light and dark conditions followed by that under 9 h light conditions (61.33 mm) while, minimum mean diametric growth (59.72 mm) was recorded under complete dark conditions (0 h photoperiod) which was statistically at par with that under 3 h photoperiod (60.03 mm) followed significantly by that under 6h/18 h light / dark conditions (60.68 mm). Irrespective of the photoperiods, the significantly maximum mean diametric growth (87.12 mm) was recorded after 96 h of incubation followed by that after 72 h of incubation (76.97 mm). However, significant minimum mean diametric growth (27.08 mm) was recorded after 24 h of incubation.

Body of the table reveals that significantly maximum diametric growth (94.08 mm) was recorded after 96 h of incubation under 12 h light conditions which was followed by that under 9 h photoperiod (86.17 mm) after same period of

incubation. However, minimum diametric growth (26.44 mm) was recorded under 9 h light conditions after 24 h of incubation which was statistically at par with that under 6 h (26.50 mm) and 3 h light conditions (26.92 mm) after same duration of incubation. Under rest of the photoperiods, an intermediate diametric growth was recorded after different duration of incubation. It was interesting to note that upto 24 h of incubation, no definite pattern in diametric growth of alga was recorded but after that, as the photoperiod increased, a corresponding increase in growth of alga was observed.

It is evident from the Fig. 15 that maximum area under growth curve was covered under 12h/12h light/dark conditions which decreased with the decrease in photoperiod indicating that photoperiod directly influenced the growth of the alga. Although, somewhat sigmoid curves were obtained under all the photoperiods indicating that the alga can grow well under dark as well as 3 h to 12 h light conditions.

Effect of different photoperiods on growth rate of *Cephaleuros virescens*

Growth rate of the alga under different photoperiods after different duration of incubation has been presented in Table 11. It is clear from the table that irrespective of time of observation, maximum mean growth rate (0.93 mm/h) was recorded under 12 h light conditions, while minimum mean growth rate (0.83 mm/h) was recorded under 0 h as well as 3 h light conditions which was statistically at par with that under 6 h (0.84 mm/h) and 9 h light conditions (0.85 mm/h). Irrespective of the photoperiods under study, significantly maximum mean growth rate (1.14 mm/h) was recorded between 24-48 h of incubation followed by that between 48-72 (0.94 mm/h) and 0-24 (0.92 mm/h) hour of incubation while, minimum mean growth rate (0.42 mm/h) was recorded between 72-96 h of incubation.

Body of the table reveals that significantly maximum growth rate (1.24 mm/h) was recorded under 12 h light conditions between 24-48 h of incubation, while minimum growth rate (0.35 mm/h) was recorded under 9 h light conditions between 72-96 h of incubation which was statistically at par with growth rate under 3 h (0.38 mm/h) photoperiod between same incubation period. An intermediate range of growth rate was recorded at rest of the photoperiods after different durations of incubation.

It is observed from the Fig. 16 that under 9 and 12 h photoperiod, the alga grew well up to 72 h of incubation and after that the growth declined resulting in the decline in difference in relative area under kinetic curve values. However, at rest of the photoperiods, the growth declined even after 48 h of incubation.

The colour of sporulating mycelium observed was greyish green under 12 h and 9 h photoperiod, green under 6h, dull greyish green under 3h photoperiod and brownish grey under 0h photoperiod. The different photoperiods also affected the growth pattern of the alga which was ray under 12h and 0h photoperiods but, ray as well as ring under rest of the photoperiods under investigation. Type of mycelial growth was also observed at different photoperiods under study. Cottony growth was recorded under 12 h while, flat growth under 9 h and 6 h photoperiod. However, uneven growth was observed under 3 h photoperiod while, it was found to be sparse under 0 h photoperiod (Fig. 17).

Different photoperiods also influenced the mycelial growth, rate of growth and growth pattern of *C. virescens*. However, the test pathogen grew well under all the photoperiods, 12h/12h photoperiod (light/dark conditions) being the optimum. These results are similar to the findings of Mata *et al.* (2012) [10] who reported that cultivating algae in an aerated culture and exposing the growth to a 12 hour period of day light at 12000 lux intensity were the best conditions. The findings of Al-Qasmi *et al*. (2012) [1] further supported our results, who concluded that light conditions have a direct effect on growth and photosynthesis of algae. For productive photosynthesis, algae needs a light/dark regime. It requires light for a photochemical phase to produce Nicotinamide adenine dinucleotide phosphate-oxidase (NADPH) and Adenosine triphosphate (ATP), as well as darkness for biochemical phase to synthesize essential molecules for growth. However, the growth of alga under 0 h photoperiod could be attributed to the fact that some of the nutrients were already present in medium that supported growth of alga even in the absence of light although, the growth was slow. Experimental investigations revealed that an increase in light duration is directly proportional to increase in mycelial growth of *C. virescens* (Al-Qasmi *et al.*, 2012) [1]. Therefore, it can be concluded that the culture of alga needs to be exposed to light/dark period with certain light intensity. Based on these studies, 12h/12h (light/dark conditions) photoperiod was selected as the best photoperiod for optimum mycelial growth of the test alga and used in subsequent experiments.

Table 1: Effect of different nutrient media on mycelial growth of *Cephaleuros virescens*

Nutrient medium	Diametric growth (mm) after time (h)				Overall mean
	24	48	72	96	
Potato dextrose agar	12.22	29.55	42.56	56.78	35.28
Potato sucrose agar	15.11	21.00	23.67	24.67	21.11
Bold's basal medium	5.67	10.22	17.50	28.33	15.43
Trebouxia medium	6.67	14.00	17.56	19.22	14.36
Bristol medium	5.44	6.78	16.45	20.44	12.28
Agarized host leaf extract	10.89	28.67	69.00	95.89	51.11
Overall mean	9.33	18.37	31.12	40.89	
Factors $CD_{p\geq0.05}$ $SE_{(d)}$ Nutrient medium 0.59 0.29 Interval 0.48 0.24 Nutrient medium x interval 1.18 0.48					

Table 2: Effect of different nutrient media on growth rate of *Cephaleuros virescens*

Name of media	Growth rate (mm/h) between time interval (h)				Overall mean
	0-24	24-48	48-72	72-96	
Potato dextrose agar	0.30	0.72	0.54	0.59	0.54
Potato sucrose agar	0.42	0.25	0.11	0.04	0.21
Bold's basal medium	0.03	0.19	0.30	0.45	0.24
Trebouxia medium	0.07	0.31	0.15	0.07	0.15
Bristol medium	0.02	0.06	0.40	0.17	0.16
Agarized host leaf extract	0.25	0.74	1.68	1.12	0.95
Overall mean	0.18	0.38	0.53	0.41	
Factors $CD_{p\geq0.05}$ $SE_{(d)}$ Nutrient medium 0.02 0.01 Time interval 0.02 0.01 Interaction 0.05 0.02					

Table 3: Effect of different nutrient media on cultural characteristics of *Cephaleuros virescens*

Nutrient medium	Colour of mycelium	Growth pattern	Type of growth	Gametangia like bodies
Potato dextrose agar	Dull white, turned cottony green with progression of time	Ring	Hairy, fluffy and cottony	+
Potato sucrose agar	White, turned green with progression of time	Ring	Cottony and fluffy	-
Bold's basal medium	Transparent which later turn green	Ray	Velvety, root like	++
Trebouxia medium	Dull white, very light greenish threads	Ring	Suppressed to cottony	-
Bristol medium	Very light transparent	Ray	Hairy threads	-
Agarized host leaf extract	Dark to light transparent, turned green	Ring as well as ray	Suppressed	+++

Table 4: Effect of different temperature regimes on the mycelial growth of *Cephaleuros virescens*

Temperature (℃)	Diametric growth (mm) after time (h)				Overall mean
	24	48	72	96	
17	5.00	9.89	18.78	35.00	17.17
20	6.67	21.00	51.67	74.67	38.50
22	9.56	32.67	59.78	88.74	47.69
25	11.33	36.67	67.22	94.56	52.44
28	10.89	34.11	67.22	94.78	51.75
30	9.55	29.33	64.22	86.78	47.47
32	6.78	23.67	57.55	80.67	42.17
Overall mean	8.54	26.76	55.21	79.31	
Factors $CD_{p\geq0.05}$ $SE_{(d)}$ Temperature 0.49 0.25 Time interval 0.37 0.19 Interaction 0.99 0.49					

Table 5: Effect of different temperature regimes on growth rate of *Cephaleuros virescens*

Temperature (℃)	Growth rate (mm/h) between interval (h)				Overall mean
	0-24	24-48	48-72	72-96	
17	0.00 (1.01)	0.41 (1.19)	0.37 (1.17)	0.67 (1.29)	0.36 (1.16)
20	0.07 (1.03)	0.60 (1.26)	1.28 (1.51)	0.96 (1.40)	0.73 (1.30)
22	0.19 (1.09)	0.96 (1.40)	1.13 (1.46)	1.21 (1.49)	0.87 (1.36)
25	0.26 (1.12)	1.06 (1.43)	1.27 (1.51)	1.14 (1.46)	0.93 (1.38)
28	0.25 (1.12)	0.97 (1.40)	1.38 (1.54)	1.15 (1.47)	0.93 (1.38)
30	0.19 (1.09)	0.83 (1.35)	1.45 (1.57)	0.94 (1.39)	0.85 (1.35)
32	0.07 (1.04)	0.70 (1.31)	1.41 (1.55)	0.96 (1.40)	0.79 (1.32)
Overall mean	0.15 (1.07)	0.79 (1.34)	1.19 (1.47)	1.01 (1.42)	
Factors $CD_{p\geq0.05}$ $SE_{(d)}$ Temperature 0.01 0.005 Time interval 0.01 0.004 Interaction 0.02 0.01					

Figures in parentheses represent (n+0.01) square root transformed values

Table 6: Effect of different temperature regimes on cultural characteristics of *Cephaleuros virescens*

Temperature (℃)	Colour of mycelium	Growth pattern	Type of growth
17	Cottony white	Ring	Hairy, fluffy and filamentous
20	Transparent to transparent white turning green and sporulating	Ring as well as ray	Suppressed
22	Transparent white to light green and sporulating at the centre	Ring	Fluffy
25	Transparent white mycelium turning green at the centre making zones in the nutrient media	Ring	Fluffy
28	Transparent white mycelium turning grey in the centre	Ring	Suppressed and smooth
30	Whitish grey	Ring	Fluffy to cottony, irregular in shape
32	Whitish grey	Ring	Fluffy to cottony, irregular and uneven

Table 7: Effect of different pH levels on mycelial growth of the *Cephaleuros virescens*

pH	Diametric growth (mm) after time (h)				Overall mean
	24	48	72	96	
6.0	5.83	33.67	62.00	87.00	47.12
6.5	9.78	37.33	63.67	86.00	49.19
7.0	11.22	39.56	70.70	87.00	52.12
7.5	12.56	39.33	71.67	89.56	53.28
8.0	11.26	39.81	73.00	89.45	53.38
8.5	11.67	40.11	72.78	89.00	53.39

9.0	9.55	37.93	71.33	89.26	52.02
Overall Mean	10.27	38.25	69.31	88.18	
Factors $CD_{p\geq0.05}$ $SE_{(d)}$ pH 0.53 0.26 Time interval 0.40 0.20 Interaction 1.06 0.53					

Table 8: Effect of different pH levels on growth rate of *Cephaleuros virescens*

pH	Growth rate (mm/h) between interval (h)				Overall mean
	0-24	24-48	48-72	72-96	
6.0	0.03	1.16	1.18	1.04	0.85
6.5	0.20	1.15	1.10	0.93	0.84
7.0	0.26	1.18	1.30	0.68	0.85
7.5	0.32	1.12	1.35	0.74	0.88
8.0	0.26	1.19	1.38	0.68	0.88
8.5	0.28	1.18	1.36	0.67	0.87
9.0	0.19	1.18	1.39	0.75	0.88
Overall mean	0.22	1.17	1.30	0.79	
Factors $CD_{p\geq0.05}$ $SE_{(d)}$ pH 0.02 0.01 Time interval 0.02 0.01 Interaction 0.04 0.02					

Table 9: Effect of different pH levels on cultural characteristics of *Cephaleuros virescens*

pH	Colour of mycelium	Growth pattern	Type of growth
6.0	Dull white to greenish grey	Ring	Sparse, cottony
6.5	Dull white to greenish grey	Ring	Sparse, cottony
7.0	Dull white to greenish grey	Ring	Suppressed, cottony
7.5	Dull white to greenish grey	Ring	Suppressed, cottony
8.0	Dull white to greenish grey	Ring	Suppressed, cottony
8.5	Cottony white to greyish green	Even	Fluffy, cottony Smooth
9.0	Greyish green	Ring	Suppressed, cottony

Table 10: Effect of different photoperiods on mycelial growth of *Cephaleuros virescens*

Photoperiod (h)	Diametric growth (mm) after time (h)				Overall mean
	24	48	72	96	
0	27.89	52.00	74.23	84.75	59.72
3	26.50	53.52	74.58	85.50	60.03
6	26.92	54.77	75.94	85.08	60.68
9	26.44	54.80	77.92	86.17	61.33
12	27.67	57.50	82.17	94.08	65.35
Overall mean	27.08	54.52	76.97	87.12	
Factors $CD_{p\geq0.05}$ $SE_{(d)}$ Photoperiod 0.52 0.26 Time interval 0.46 0.23 Interaction 1.03 0.51					

Table 11: Effect of different photoperiods on growth rate of *Cephaleuros virescens*

Photoperiod (h)	Growth rate (mm/h) between interval (h)				Overall mean
	0-24	24-48	48-72	72-96	
0	0.95	1.01	0.93	0.43	0.83
3	0.92	1.16	0.88	0.38	0.83
6	0.90	1.13	0.88	0.46	0.84
9	0.90	1.18	0.97	0.35	0.85
12	0.94	1.24	1.03	0.50	0.93
Overall mean	0.92	1.14	0.94	0.42	
Factors $CD_{p\geq0.05}$ $SE_{(d)}$ Photoperiod 0.03 0.01 Time interval 0.02 0.01 Interaction 0.05 0.03					

Table 12: Effect of different photoperiods on cultural characters of *Cephaleuros virescens*

Photoperiod (h)	Colour of sporulating mycelium	Growth pattern	Type of growth
12	Greyish green	Ray	Cottony
9	Greyish green	Ray and ring	Flat
6	Green	Ray and ring	Flat
3	Dull greyish green	Ray and ring	Uneven, cottony as well as sparse
0	Brownish Grey	Ray	Sparse

Fig 1: Symptoms of red rust of litchi under field conditions

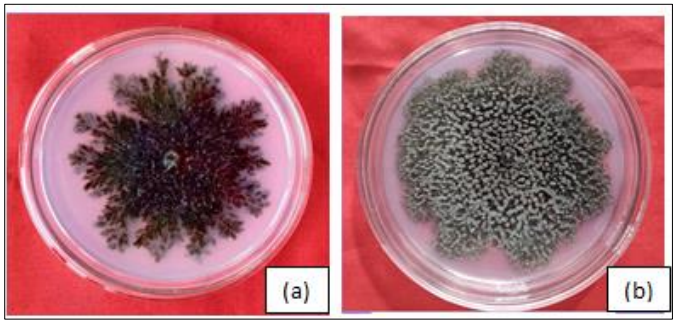

Fig 2: (a) Pure culture of *Cephaleuros virescens* (b) Formation of gametangia- like bodies in pure culture

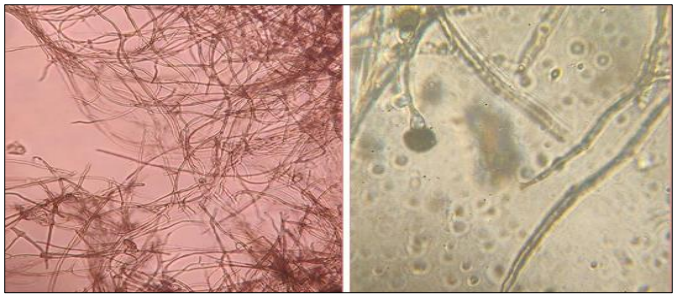

Fig 3: Mycelium, sporangia and gametangia-like bodies in pure culture

Fig 4: Symptoms of red rust of litchi under laboratory conditions

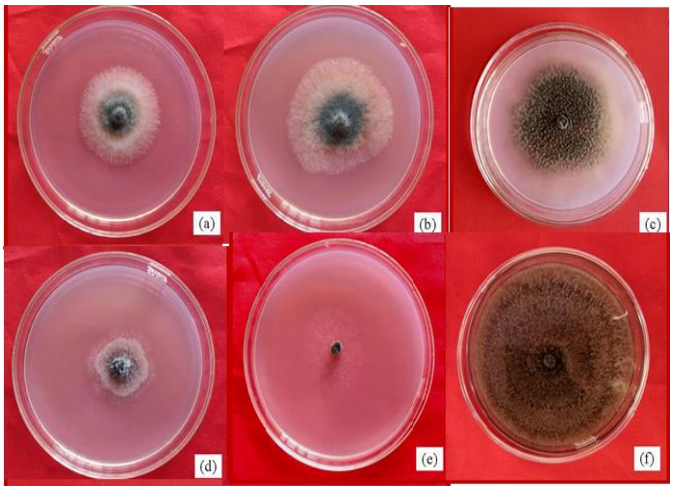

Fig 5: Growth pattern of *Cephaleuros virescens* on different nutrient media (a) PDA (b) PSA (c) BBM (d) Trebouxia (e) Bristol (f) Agarized HLE

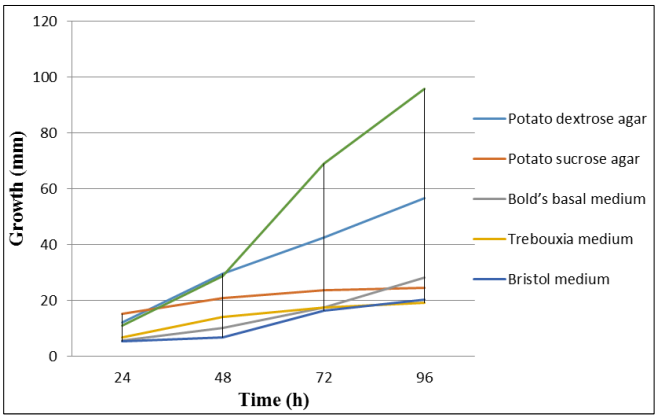

Fig 6: Growth of *Cephaleuros virescens* under the influence of different nutrient media

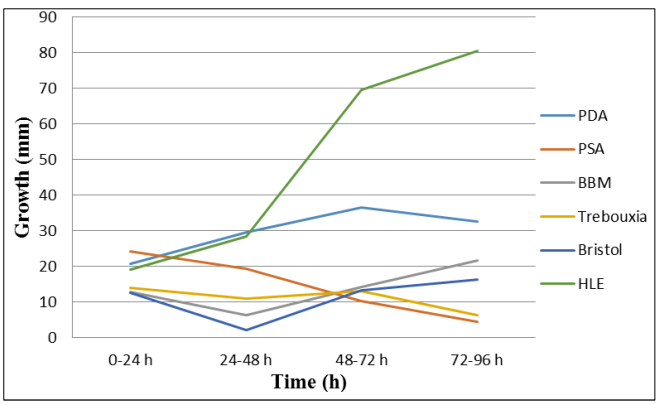

Fig 7: Difference in relative area under kinetic curve values plotted against time to depict the effect of nutrient media on growth of *Cephaleuros virescens*

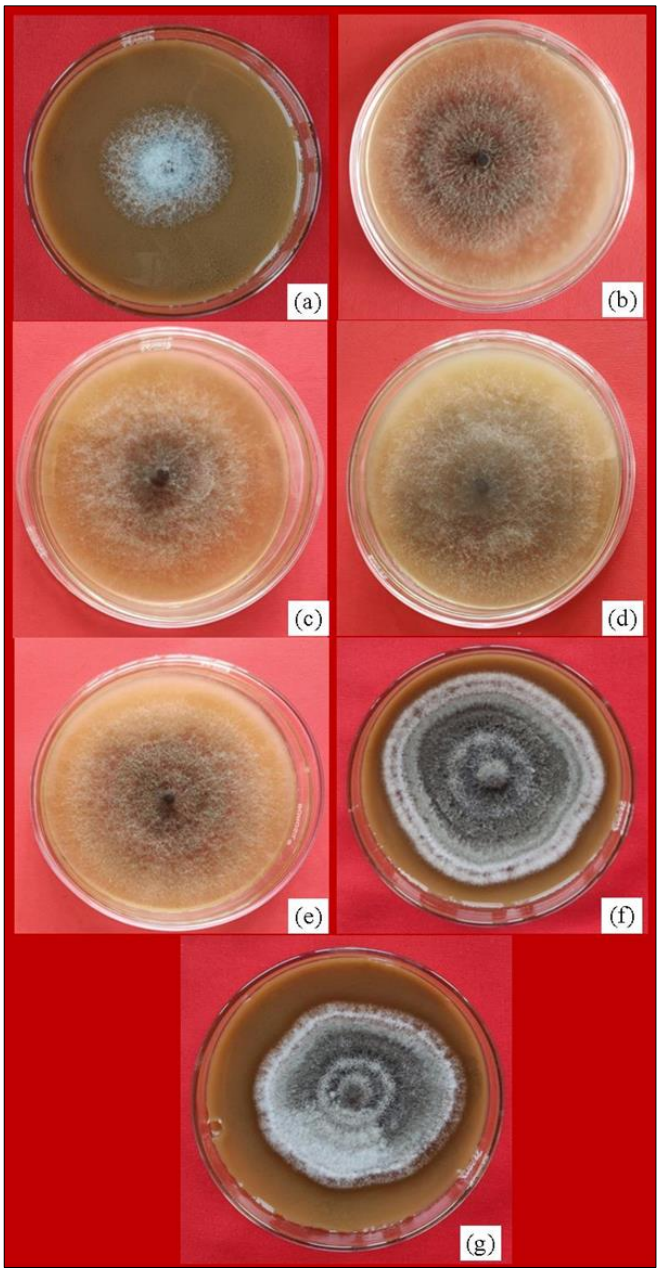

Fig 8: Mycelial growth of *Cephaleuros virescens* at different temperatures (a) 17 (b) 20 (c) 22 (d) 25 (e) 28 (f) 30 (g) 32 ℃

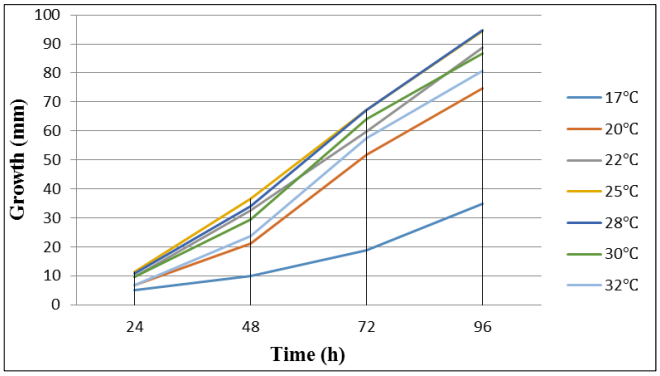

Fig 9: Growth curves of *Cephaleuros virescens* under the influence of different temperature regimes

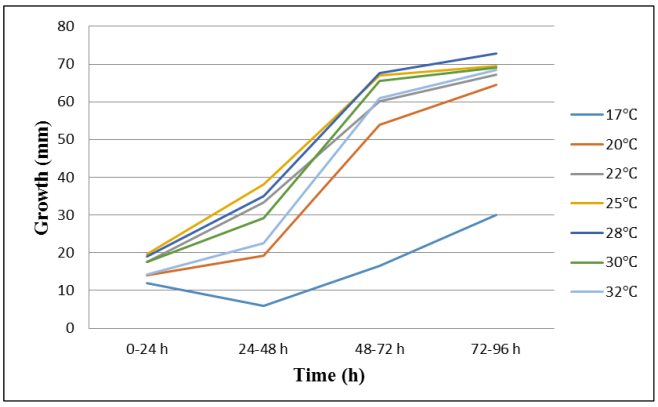

Fig 10: Difference in relative area under kinetic curve values plotted against time to depict the effect of different temperatures on growth of *Cephaleuros virescens*

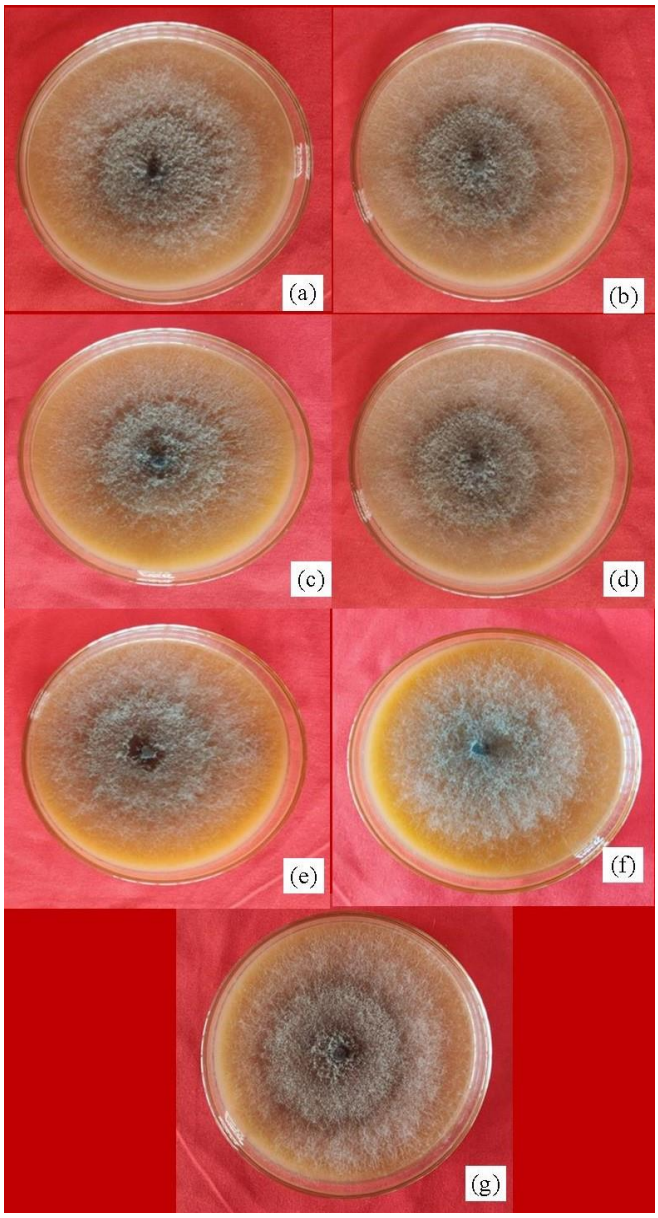

Fig 11: Mycelial growth of *Cephaleuros virescens* at different pH levels (a) 6.0 (b) 6.5 (c) 7.0 (d) 7.5 (e) 8.0 (f) 8.5 (g) 9.0

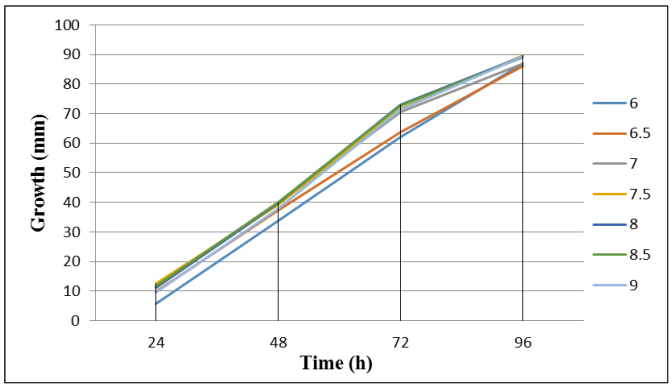

Fig 12: Growth curves of *Cephaleuros virescens* under the influence of different pH levels

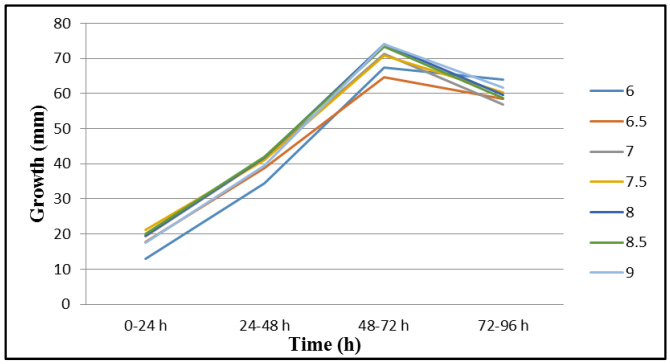

Fig 13: Difference in relative area under kinetic curve values plotted against time to depict the effect of different pH levels on growth of *Cephaleuros virescens*

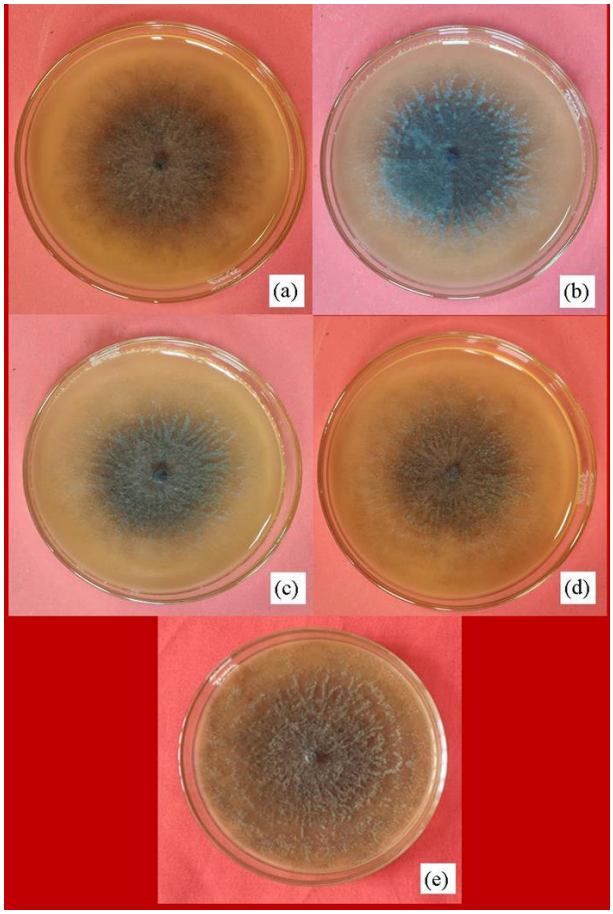

Fig 14: Mycelial growth of *Cephaleuros virescens* at different photoperiods (a) 0 h (b) 3 h (c) 6 h (d) 9 h (e) 12 h

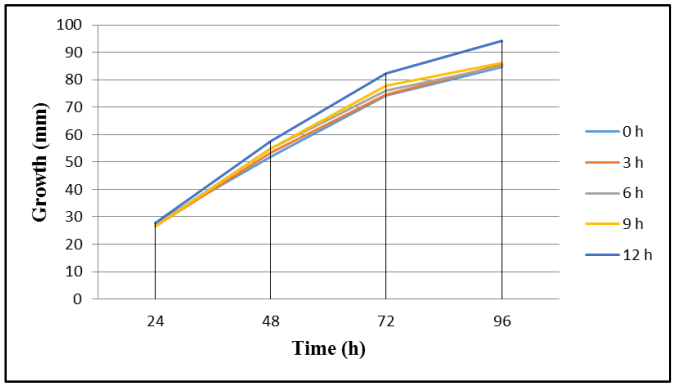

Fig 15: Growth curves of *Cephaleuros virescens* as influenced by different photoperiods

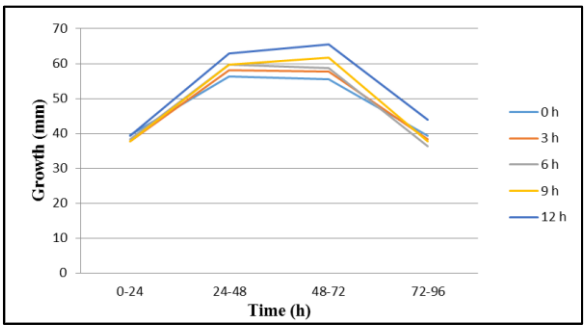

Fig 16: Difference in relative area under kinetic curve values plotted against time to depict the effect of different photoperiods on growth of *Cephaleuros virescens*

Conflict of Interest

The Author(s) declare(s) that there is no conflict of interest.

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