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In vitro assessment of the influence of growing media, specific pH and temperature levels on the growth of *Ceratocystis fimbriata*, instigating wilt of pomegranate

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Abstract
In vitro evaluation studies were conducted to investigate the influence of five Non synthetic/semi synthetic media and three synthetic media on the growth of *Ceratocystis fimbriata*. In addition to effect of solid media, nine different levels of hydrogen-ion concentration (pH) and seven levels of temperatures were also evaluated to record the growth of *Ceratocystis fimbriata*, causing wilt of pomegranate. After a period of 120 hours of inoculation, it was noted that Potato dextrose agar was most suitable medium for the growth of *Ceratocystis fimbriata*. Potato dextrose agar supported quicker perithecium development than other media. Perithecium also developed in case of Malt agar, Oat meal agar and Richard's agar. A pH level of 7.5 was deemed as the optimum pH for the growth as well as sporulation of test fungus and temperature of 30 °C was recorded to be the best for the growth of *Ceratocystis fimbriata.*

Keywords: *Ceratocystis fimbriata*, Pomegranate Wilt, Temperature and Hydrogen-ion concentration (pH)

1. Introduction
Pomegranate botanically described as *Punica granatum* L. is accorded to family Punicaceae constituting of two species, *P. protopunica Balf. f. 1882* and *Punica granatum* L. *Punica protopunica* is the only affiliated relative of *P. granatum* species which is forthwith cultivated with it being endemic to Yemen (Levin, 2006) [12]. Pomegranate is deemed to be indigenous to Iran (Stover and Mercure, 2007) [20] and is also recognised to be native to Turkey (Ercisli *et al.*, 2007) [4]. *Punica protopunica* was proposed as the origin of the genus described as *Punica* on the foundation of xylem anatomy (Shilkina, 1973) [17]. Pomegranate is cultivated commercially in India, Turkey, Iran, Tunisia, Spain, Afghanistan, Morocco, China, Japan, Greece, Armenia, France, Italy and Egypt. The designate 'pomegranate' is established on the Latin title of ' grainy apple,' that is, *Malum granatum*. The collective term *Punica* is attributed to *Pheonicia* restricted in *Punicaceae* family.
During the years 2018-2019 India had an area of 2,46,000 hectare under pomegranate cultivation with a production of 28,65,000 MT (National Horticulture Board, 2019). India is the only nation in the globe where pomegranate is accessible all year round (January-December). Among India's various pomegranate-growing areas, Maharashtra is the country's biggest crop producing state occupying 2/3 of total area followed by Gujarat, Karnataka, Andhra Pradesh, Madhya Pradesh and Rajasthan.
The production of pomegranate is associated with various issues. Biotic constraints affecting pomegranate are associated with problems of disease and pests of which, *Ceratocystis fimbriata* is the incitant of pomegranate wilt and *Xanthomonas axonopodis* pv *punicae* is known to cause bacterial blight disease and as a result were proclaimed as major scourges of pomegranate. Wilt has been defined as a significant biotic restriction of pomegranate in India (Somasekhara and Wali, 1999) [18] Other Asian countries from where wilt of pomegranate has been reported are Pakistan (Fateh *et al.*, 2006) [5] and China (Huang *et al.*, 2003) [9]. Karnataka, Maharashtra, Tamil Nadu, Gujarat and Andhra Pradesh are regarded as hot spots for wilt in India (Somasekhara *et al.*, 2000) [19].
Commencing disease symptoms associated with *Ceratocystis fimbriata* which appear on the plants emerge as yellowing followed by drooping of the leaves on one branch generally on the

upper crown resulting in senescence and further spread to the entire plant. The fungus survives for almost 2 weeks in the soil and in decaying tree components for up to 30 months (Grosclaude *et al.*, 1988; 1995) [6, 7]. Infectious agent survival is accelerated by the production of various chlamydospores that are dense asexual spores and other microconidia. *Ceratocystis* spp. infection may happen through the crown as well as via roots. The roots penetration is autonomous of the injuries that lead to rapid plant death. In the event that infection occurs through the crown, the disease starts in lateral branches, advances smoothly towards the trunk, enters the trunk and eventually triggers the plant's mortality. The pathogen dispersion happens primarily through infected cuttings, farm equipments or through infected field soil.

Incidence of pomegranate wilt was recorded by Somasekhara *et al.* (2000) [19] and it was stated that pomegranate was heavily impacted by wilt disease, and the wilting severity is rising at a quicker pace day in and day out, as 6,745 plants wilted out of 54,866 plants sampled at 128 sites, resulting in financial losses of Rs. 67, 45 lakhs throughout the years from 1996 to 1999. Wilt of pomegranate is a burning issue in Karnataka and can become an epiphytotic disease in a short time.

Keeping in view of these criterions, the present investigations on wilt disease of pomegranate were therefore, directed to elucidate some of the critical aspects of survival ability of test fungus in accordance to different media, specific levels of hydrogen-ion concentration (pH) and temperature.

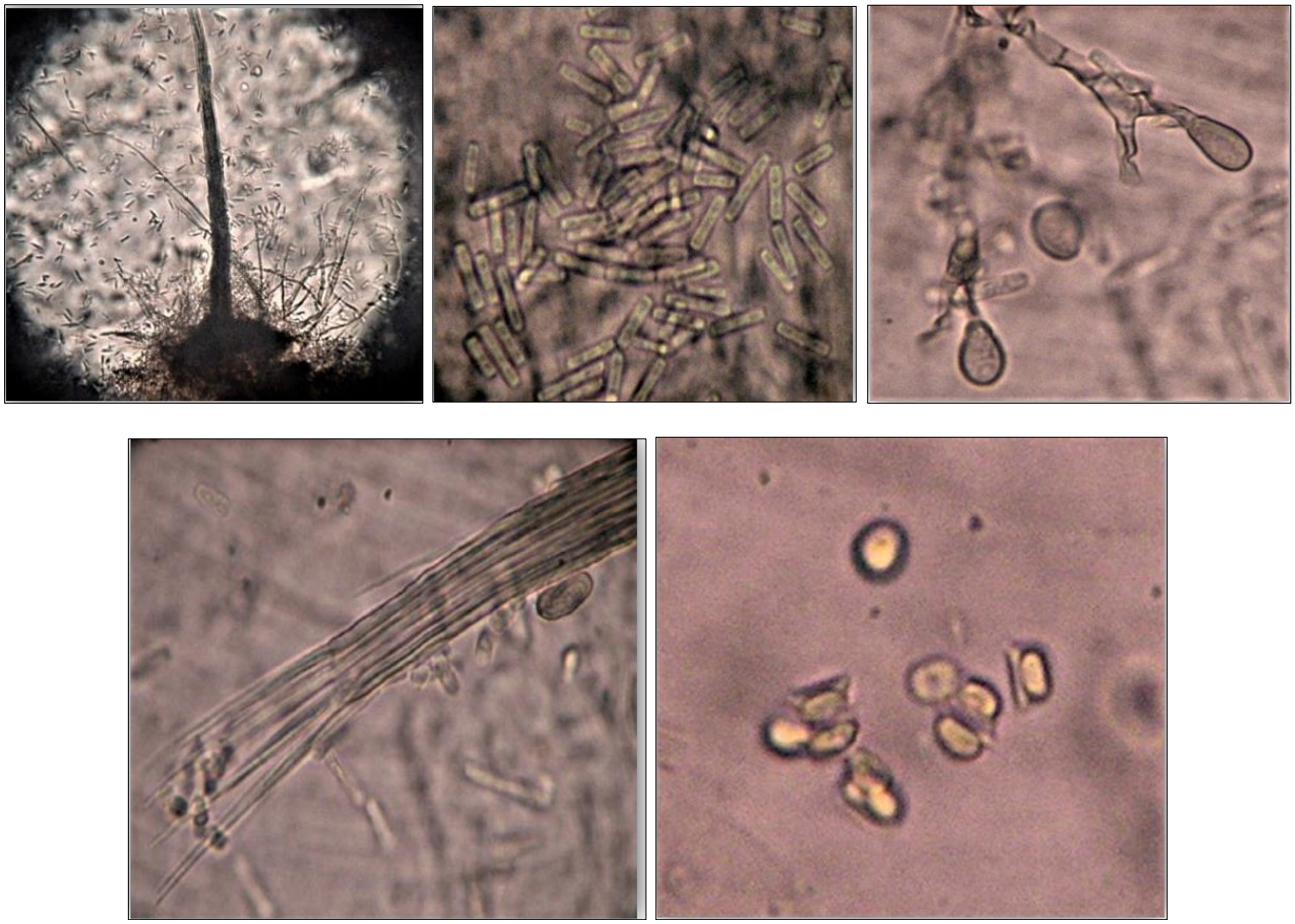
2. Materials and Methods

2.1 Isolation of test fungus

Following the conventional method of tissue isolation, the isolation of fungus was carried out. The brownish discolored root pieces of pomegranate were sterilized in 1:1000 solution of mercuric chloride for 30 seconds along with some healthy parts and rinsed thrice carefully in sterile distilled water to prevent residues of mercuric chloride, if any. Bits of root were then transmitted to Petri plates containing sterilised Potato dextrose Agar. At 25±2°C, the Petri plates were incubated in the BOD incubator at 25±2ºC and periodically monitored for fungal growth. The fungal colonies that emerged from the bits were moved to slants of Potato Dextrose Agar and were put in an incubator at for 15 days at ambient room temperature.

2.2 Identification of the fungus

To verify the identity/individuality of the fungus, the ascospores and perithecia from roots of the infected pomegranate plant were inspected under the high-power (40X) microscope. The morphological characters of conidia, conidiophores perithecia and ascospores were compared with those described by Khosla *et al.*, (2011) to establish the distinctiveness of the pathogen (Figure 1).



a = Black Perithecia with globose base and long neck
b = Hyaline cylindrical Conidia
c = Thick walled aleuroconidia on specialised conidiophore
d = Ascospore discharge from perithecial neck
e = Small, hyaline and hat shaped ascospores

Fig 1 (a-e): Microscopic examination of isolated fungus

2.3 Maintenance of pathogen culture
The developed cultures were regularly sub-cultured on slants containing Potato Dextrose Agar and incubated at a temperature of 25±2 ºC for 15 days in the BOD incubator. Such slants of mother culture were set aside in the refrigerator at 4 °C for further use.

2.4 Influence of Media on the growth of *Ceratocystis fimbriata*
It is necessary to carry out thorough cultural studies of *Ceratocystis fimbriata* with respect to nutritional and physiological characteristics in order to establish good management strategies. The investigations were therefore, aimed at finding the nutritional and physiological requirements of the pathogen. Growth of *Ceratocystis fimbriata* was evaluated on following synthetic and semi synthetic/ non synthetic media.

2.4.1 Non synthetic or semi synthetic media
1. Potato dextrose agar
2. Host leaf extract agar
3. Oat meal agar
4. Corn Meal Agar
5. Malt extract agar

2.4.2 Synthetic media
1. Czapeck's (dox) agar
2. Richard's agar
3. Sabouraud's Agar

The formulation and methodology of preparation of media as mentioned above were attained from Ainsworth and Bisby's 'Dictionary of the Fungi' by Hawksworth *et al*. (1983) [8].

2.5 Physiological studies
To carry out the physiological studies, *Ceratocystis fimbriata* cultures were grown in PDA medium in sterlised Petri plates. Twenty ml of each media under observation was poured separately into Petri plates and were incubated for 15 days at 25±2^{0}C after inoculating each plate with 7 days old 5 mm culture disc of *Ceratocystis fimbriata* under aseptic conditions with the use of 5 mm cork borer. The fungus was avowed to develop and diametric growth was recorded at an interval of 24, 48, 72, 96 and 120 hours after inoculation. An average of 3 replications in a treatment was worked out for comparing growth under different treatments. Results were figured out statistically and compared by making use of Analysis of Variance (ANOVA).

2.6 Effect of pH on growth of *Ceratocystis fimbriata*
The influence of pH on the intensification of *Ceratocystis fimbriata* was studied at nine pH levels *viz.*, pH of 5.0, pH of 5.5, pH of 6.0, pH of 6.5, pH of 7.0, pH of 7.5, pH of 8.0, pH of 8.5 and pH of 9.0. pH of the PDA medium was regulated to different pH levels with NaOH having normality 1.0 or HCl with normality 1.0 by using pH meter and sterilized at 121ºC for 20 minutes. Twenty ml of PDA media was then poured separately in each 90 mm autoclaved Petri plates and was used as basal medium. Petri plates were inoculated with 7 day's old 5 mm disc of *Ceratocystis fimbriata* under aseptic conditions and incubated at a temperature (25±2ºC). Diametric growth was recorded at an interval of 24, 48, 72, 96 and 120 hours. Each treatment was having 3 numbers of replications. Diametric growth (mm) was recorded along with growth rate (mm/hr). Average value of the replications in a treatment was worked out and used as a quantitative measure for comparing the growth under different treatments. Results were analyzed statistically and compared by making use of Analysis of Variance (ANOVA).

2.7 Effect of temperature on growth of *Ceratocystis fimbriata*
The consequence of temperature on the intensification of *Ceratocystis fimbriata* was studied at seven temperature levels *viz*; 10 °C, 15 °C, 20 °C, 25 °C, 30 °C, 35 °C and 40 °C. Twenty ml of PDA media was poured separately in each 90 mm sterilised Petri plates. Each treatment had 3 replications. Five mm culture discs of 7 day's old actively growing mother culture of *Ceratocystis fimbriata* were positioned in the core of Petri plates and incubated at different temperature levels. Diametric growth (mm) was recorded along with growth rate (mm/hr) at an interval of 24, 48, 72, 96 and 120 hours Average value of the replication in a treatment was worked out and used as a quantitative measure for comparing the growth under different treatments. Results were figured out statistically and compared by making use of Analysis of Variance (ANOVA).

3. Results
3.1 Growth of *Ceratocystis fimbriata* on different media
The growth characters of *Ceratocystis fimbriata* were studied on eight different media as described in "Material and Methods" section. The results of growth of *Ceratocystis fimbriata* are placed in Table 1. The results pointed out to the conclusion that both radial colony diameter and production of perithecium of *Ceratocystis fimbriata* revealed notable variation when grown on different media. When mean diametric growth of *C. fimbriata* was calculated among the eight media, PDA (15.86 mm) was pursued by Oat Meal Agar (15 mm) which was statistically at par with Richard's Agar (14.86 mm). Corn Meal Agar was the next best medium supporting the colony growth (13.80 mm) and was followed by Czapek's (dox) Agar (10.53 mm). Growth on Malt extract Agar (9.66 mm) was closely followed by Sabouraud's Agar in terms of supporting radial growth of *Ceratocystis fimbriata* (8.80 mm) and lowest growth was noted in case of Host leaf extract (2.60 mm).
Maximum growth rate was noted in PDA (0.23 mm/hr) followed by Corn meal agar (0.22 mm/hr) and Richard's Agar (0.22 mm/hr) which were statistically at par with each other followed by Oat meal agar (0.21 mm/hr). Malt extract agar (0.17 mm/hr) and Czapek's (dox) agar (0.17 mm/hr) were at par with each other pursued by Sabouraud's agar (0.16 mm/hr). Least growth rate was noted in case of Host leaf extract (0.06 mm/hr). PDA supported quicker perithecium development than other media. Perithecium also developed in case of Malt agar, Oat meal agar and Richard's agar. In the case of PDA, abundant mycelial development was noted which was consistently white, equally dense, cottony mycelium appearance at the outer rim, very notable pin head structures and undulating margin of the colony was also noted.

3.2 Effect of pH on growth of *Ceratocystis fimbriata*
The data recorded and placed in Table 2 revealed that the fungus grew well at all pH levels investigated except for pH 5.0. Maximum average diametric growth after a period of 120 hours from date of inoculation was recorded at a pH of 7.5 (16.33 mm), pursued by a pH of 7.0 (15.53 mm) and pH of 8.00 (15.53 mm), which were at par. Followed by a pH of

8.50 with 14.66 mm radial growth. The growth of *Ceratocystis fimbriata* lowered from pH 5.5 (5.80 mm) downwards and also at pH 9.0 (5.86 mm) which were noted to be at par statistically. Least growth was noted at pH 5.0 (3.13 mm).

Maximum growth rate was noted at a pH of 7.5 (0.22 mm/hr) and 8.5 (0.22 mm/hr) which were noted to be at par statistically pursued by a pH of 7.0 and 8.0 where growth rate was 0.20 mm/hr respectively and were statistically at par with each other. Next best hydrogen-ion concentrations were 6.5 (0.16 mm/hr), 6.0 (0.15 mm/hr) and 9.0 (0.13 mm/hr) followed by a pH of 5.5 (0.12 mm/hr). Least growth of *C. fimbriata* was noted at a pH of 5.0 with a growth rate of 0.09 mm/hr after a period of 120 hours of inoculation.

3.3 Effect of temperature on growth of *Ceratocystis fimbriata*

The impact of temperature levels on the growth of *Ceratocystis fimbriata* was studied as explained in "Material and Methods" and the results are shown in Table 3. The average maximum growth of the fungus *Ceratocystis fimbriata* (16.13 mm) was noted at incubation temperature of 30°C after five days of inoculation. Growth at 25°C (15 mm) was found to be followed by a temperature of 20°C (13.40 mm) and at 35°C (12.13 mm). The fungus grew very little at 15°C (1.86 mm), 40°C (1.86 mm) and least growth of *C. fimbriata* was noted at 10°C (1.66 mm). Also, the temperatures of 10°C, 15°C and 40°C were noted to be statistically at par with each other.

Maximum growth rate was noted at a temperature of 30 °C (0.24 mm/hr) followed by a temperature of 25 °C (0.20 mm/hr). Next best studied temperatures were 20 °C (0.18 mm/hr) and 35 °C (0.17 mm/hr) which were at par with each other. The least growth rate was noted at temperatures of 10 °C, 15 °C and 40 °C which were noted to be statistically at par with similar growth rate of 0.03 mm/hr after a period of 120 hours of inoculation.

Table 1: Effect of different media on the growth of *Ceratocystis fimbriata*

S. No.	Medium	Diametric Growth (mm) after hours						Growth Rate (mm/h) after hours					
		24	48	72	96	120	Mean (Medium)	24	48	72	96	120	Mean
Non-synthetic/Semi Synthetic Media													
1.	Potato Dextrose Agar (PDA)	3.33	11.33	17.00	21.33	26.33	15.86^{a}	0.33	0.23	0.18	0.20	0.24	0.23^{a}
2.	Host-Leaf Extract	0.00	0.66	2.33	3.66	6.33	2.60^{g}	0.02	0.06	0.05	0.11	0.07	0.06^{f}
3.	Oat Meal Agar	2.33	9.33	17.33	21.66	24.33	15.00^{b}	0.29	0.33	0.18	0.11	0.17	0.21^{c}
4.	Corn Meal Agar	2.00	7.33	15.33	19.66	24.66	13.80^{c}	0.22	0.33	0.18	0.20	0.16	0.22^{b}
5.	Malt Extract Agar	0.33	5.00	10.66	14.33	18.00	9.66^{e}	0.19	0.23	0.15	0.15	0.12	0.17^{d}
Synthetic Media													
6.	Czapek's (dox) Agar	1.00	6.00	11.66	15.33	18.66	10.53^{d}	0.20	0.23	0.15	0.13	0.16	0.17^{d}
7.	Richard's Agar	2.33	9.66	16.66	21.00	24.66	14.86^{b}	0.30	0.29	0.18	0.15	0.20	0.22^{b}
8.	Sabouraud's Agar	0.33	4.33	9.33	13.33	16.66	8.80^{f}	0.16	0.20	0.16	0.13	0.18	0.16^{e}
	Mean (Time Interval)	1.45	6.70	12.54	16.29	19.95		0.21	0.24	0.15	0.15	0.16	
Effect							C.D.$_{0.05}$						C.D.$_{0.05}$
Media							0.74						0.004
Time Interval							0.58						0.003
Media x Time Interval							1.66						0.009

Table 2: Effect of hydrogen–ion concentration (pH) on the growth of *Ceratocystis fimbriata*

S. No.	pH of the Medium	Diametric Growth (mm) after hours						Growth Rate (mm/h) after hours					
		24	48	72	96	120	Mean	24	48	72	96	120	Mean
1.	5.0	0.00	0.00	2.66	4.33	8.66	3.13^{g}	0.03	0.11	0.06	0.18	0.09	0.09^{g}
2.	5.5	0.00	1.66	6.33	8.33	12.66	5.80^{f}	0.06	0.19	0.08	0.18	0.11	0.12^{f}
3.	6.0	0.66	4.66	10.33	11.66	15.66	8.60^{e}	0.16	0.23	0.05	0.16	0.13	0.15^{d}
4.	6.5	2.33	6.66	12.66	17.33	19.00	11.60^{d}	0.18	0.25	0.19	0.06	0.10	0.16^{c}
5.	7.0	3.33	11.33	17.00	21.33	24.66	15.53^{b}	0.33	0.23	0.18	0.13	0.15	0.20^{b}
6.	7.5	3.33	12.66	17.33	21.66	26.66	16.33^{a}	0.38	0.19	0.18	0.20	0.17	0.22^{a}
7.	8.0	2.66	13.33	16.33	20.66	24.66	15.53^{b}	0.44	0.12	0.18	0.16	0.12	0.20^{b}
8.	8.5	1.66	12.33	14.33	20.33	24.66	14.66^{c}	0.44	0.08	0.25	0.18	0.14	0.22^{a}
9.	9.0	0.33	1.33	5.66	8.66	13.33	5.86^{f}	0.04	0.18	0.12	0.19	0.11	0.13^{e}
	Mean	1.59	7.11	11.40	14.92	18.88		0.23	0.17	0.14	0.16	0.12	
Effect							**C.D.$_{0.05}$**						**C.D.$_{0.05}$**
pH							0.59						0.004
Time Interval							0.44						0.003
pH x Time Interval							1.33						0.009

Table 3: Effect of temperature on the growth of *Ceratocystis fimbriata*

S. No.	Temperature (°C)	Diametric Growth (mm) after hours						Growth Rate (mm/h) after hours					
		24	48	72	96	120	Mean	24	48	72	96	120	Mean
1.	10	0.00	1.66	2.00	2.33	2.33	1.66^{e}	0.06	0.01	0.01	0.03	0.03	0.03^{d}
2.	15	0.00	2.00	2.00	2.66	2.66	1.86^{e}	0.08	0.03	0.02	0.03	0.01	0.03^{d}
3.	20	1.66	10.66	14.33	18.66	21.66	13.4^{c}	0.37	0.15	0.18	0.12	0.11	0.18^{c}
4.	25	2.33	12.33	16.33	20.33	23.66	15.00^{b}	0.41	0.16	0.16	0.09	0.18	0.20^{b}
5.	30	2.66	12.66	17.66	21.33	26.33	16.13^{a}	0.41	0.20	0.15	0.20	0.24	0.24^{a}
6.	35	1.33	9.33	13.33	17.33	19.33	12.13^{d}	0.33	0.16	0.16	0.08	0.12	0.17^{c}
7.	40	0.00	2.00	2.33	2.33	2.66	1.86^{e}	0.08	0.01	0.03	0.01	0.03	0.03^{d}
	Mean	1.14	7.23	9.71	12.14	14.09		0.25	0.10	0.10	0.08	0.10	

Effect	C.D.$_{0.05}$		C.D.$_{0.05}$
Temperature	0.58		0.018
Time Interval	0.49		0.015
Temperature x Time Interval	1.30		0.04

4. Discussion

4.1 Growth of *Ceratocystis fimbriata* on different media

Many researchers have previously produced findings similar to our current investigations as Kiryu (1939) [11] recorded *Ceratocystis paradoxa*'s diverse development in distinct media and noted that development on PDA had been sumptuous, Sastry *et al.* (1988) [15] revealed that all three isolates of *Ceratocystis paradoxa* from areca nut, banana and sugarcane isolates sporulated and grew well on semi-synthetic medium and Sastry *et al.* (1989) [16] revealed that Potato Dextrose Agar was the most suited media for development of *C. paradoxa*. The pathogen's next favored media was oat meal agar. Also Yadahalli (2005) [21] noted abundant sporulation when *C. paradoxa* was cultivated on PDA. Each organism has a clear model in which it achieves peak development up to a certain period of time and thereafter growth rate and subsequently decreases. In this research, after five days of inoculation in Potato Dextrose Agar, *Ceratocystis fimbriata* achieved peak development and the findings are in accordance with the observations made by Yadahalli (2005) [21].

4.2 Effect of pH on growth of *Ceratocystis fimbriata*

The results of our study concur with the results of Kiryu (1939) [11] who revealed that the optimum pH for *Ceratocystis paradoxa* was 5.5-6.3 and that comparatively robust development of fungus happened around 5.0-7.0 in the pH spectrum and Chi (1949) [3] recorded optimum *Ceratocystis paradoxa* development when it was grown at a hydrogen-ion concentration of 5.0 to 7.0. Also Adisa (1985) [2] concluded that when *C. paradoxa* was isolated from pineapple fruit and cultured, the study revealed optimum development of mycelium at pH 6.0. Whereas, Joshi (1999) [10] noted that *C. paradoxa* could thrive in a broad spectrum of pH 4-8, but optimum pH was 6.0 and Yadahalli (2005) [21] described that Hydrogen ion concentration set at 6.5 resulted in abundance growth of *Ceratocystis paradoxa*. The findings also establish the dominance of PDA medium for development of the *C. fimbriata* at different levels of pH.

4.3 Effect of temperature on growth of *Ceratocystis fimbriata*

The findings of our current investigations are in alignment with those of Milanes *et al.* (1986) [13] who, while studying the consequence of temperature on intensification of the fungus revealed that with a maximum of 34 °C and a minimum of 12 °C, 28 °C was discovered to be the most encouraging temperature for growth of mycelium of *Ceratocystis paradoxa* and when the setts of sugar cane were inoculated and incubated at different temperatures, the optimum infection temperature was 22-26 °C and a minimum of 12 °C.

In all three isolates of *C. paradoxa* from Areca nut, Coconut and Sugarcane, Sastry *et al.* (1988) [15] recorded maximum sporulation at 25 °C and 28 °C and according to Accordi (1989) [1], *Ceratocystis fimbriata* grows best at 18 °C to 28 °C and can develop ascospores within a period of 7 days.

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6. Compliance with ethical satandards

The authors are compliant with Ethical Standards put forward by the Journal and assure that there is no potential conflict of interest; also no research on human or animal subject was carried out. The manuscript has been submitted with informed consent.

7. Data availability

The datasets generated during and/ or analysed during the current study are available from the corresponding author on reasonable request.

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