Journal of Scientific Research and Reports

Volume 30, Issue 8, Page 1-19, 2024; Article no.JSRR.116995
ISSN: 2320-0227

Morpho-Molecular and Management of Pomegranate (*Punica granatum* L.) Wilt Caused by *Ceratocystis fimbriata* Ellis and Halst

Suhas Lokure [a], Somasekhara, Y, M [b++] and Ravichandra GK [c*]

[a] Department of Plant Pathology, College of Agriculture, UAS, GKVK, Bangalore-560065, India.
[b] Department of Plant Pathology, UAS, GKVK, Bangalore-560065, India.
[c] Department of Plant Pathology, College of Agriculture, KSNUAS, Shivamogga-577204, India.

Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

Article Information

DOI: https://doi.org/10.9734/jsrr/2024/v30i82220

Open Peer Review History:
This journal follows the Advanced Open Peer Review policy. Identity of the Reviewers, Editor(s) and additional Reviewers, peer review comments, different versions of the manuscript, comments of the editors, etc are available here: https://www.sdiarticle5.com/review-history/116995

Original Research Article

Received: 09/05/2024
Accepted: 11/07/2024
Published: 15/07/2024

ABSTRACT

Pomegranate wilt poses a significant threat to pomegranate production, holding considerable economic importance in the cultivated regions of pomegranates. The study investigated the wilt in pomegranate cultivation, revealing characteristic symptoms including yellowing and/or wilting of the leaves on a single branch usually in the upper crown and senescence. Brown discoloration was observed in the root, stem and branches of severely affected plants. The cross-sectioned root and stem portions of the wilted plants showed only the presence of brown discolorations in the vascular regions. The wilt incidence in Karnataka ranging from 1.14 to 62.06 Percent. PCR analysis and 18S

[++] Professor;
**Corresponding author: E-mail: ravichandragk13@gmail.com;*

Cite as: *Lokure, Suhas, Somasekhara, Y, M, and Ravichandra GK. 2024. "Morpho-Molecular and Management of Pomegranate (Punica Granatum L.) Wilt Caused by Ceratocystis Fimbriata Ellis and Halst". Journal of Scientific Research and Reports 30 (8):1-19. https://doi.org/10.9734/jsrr/2024/v30i82220.*

rDNA region was sequenced, it has been confirmed *C. fimbriata* as the causal organism for pomegranate wilt and Phylogenetic analyses placed the pomegranate isolates among members of the LAC of the *C. fimbriata* complex; specifically, to populations that appear to be native to eastern and northern South America. Optimal growth of the pathogen was observed at 30 °C with pH 7.0 and best media for culturing *C. fimbriata* is Potato dextrose agar and Potato carrot agar, *in vitro evaluation of fungicides showed* propiconazole and mancozeb highest inhibiting mycelial growth. *Trichoderma viride* (Tv-3) and *Bacillus subtilis* emerged as the most effective bio-agent, while pongamia leaf extract exhibited maximum fungal growth inhibition. These can be used for the management of wilt disease.

Keywords: Disease incidence; fungicides; botanicals; bioagents.

1. INTRODUCTION

"Pomegranate (*Punica granatum* L.*)* is belongs to the family Lythraceae or Punicaceae, subfamily *Punicoideae*, having chromosome number 2n = 16 or 18. Iran is the origin of pomegranate. Ganesh, Mridula, Arakta, Bhagwa, Kesar, G-137, and Khandar are the most common kinds for processing and table usage. Because of its hardiness, adaptability, drought tolerance, better yield levels, outstanding keeping quality and remunerative prices is obtained in both local and international markets. Hence, the area under pomegranate is rising worldwide. It grows well in the dry tropics and subtropics and thrives well on low-fertility soils, as well as being salt tolerant" [1].

Presently, it is widely cultivated, in India *i.e.,* Maharashtra, Karnataka, Andhra Pradesh, Gujarat, Rajasthan and Himachal Pradesh over an area of 264 thousand hectares with 2329 thousand MT [2] production. Maharashtra is the highest producer (147.91thousand ha area, 1789.46 thousand MT production) of pomegranate, hence this state is called as basket of pomegranate. In Karnataka, area under cultivation is 19,000 ha with a production of 2,04,000 tonnes and major growing districts are Bagalkot, Ballari, Chitradurga, Chikmagalur, Bengaluru Rural, Vijaypura and Kalburgi [3].

Pomegranate has been hailed as a super food and its nutraceutically worth as well as its versatility as a cash crop have drawn the attention of researchers, growers and consumers alike. The adaptability of the pomegranate plant to a wide range of soil, water and climatic conditions has changed the crop into a viable alternative to horticulture crops with remunerative benefits from a limited area and export possibilities. With all these prospects, pomegranate cropping is largely limited by biotic stresses s [1].

The wilt disease has been first reported on pomegranate [4] and also other parts of pomegranate growing regions including, China [5] Pakistan [6] and Costarica [7]. In Indian states *viz*., Maharashtra, Karnataka, Andra Pradesh, Tamil Nadu, Gujarat [8] and Himachal Pradesh [9] are considered to be a major hot spot for pomegranate wilt.

In 1996, the fungus *C. fimbriata* was isolated from infected plant parts. Disease is characterized by the initial symptoms on plants as yellowing on one portion of the stem and wilting of the leaves on a single branch leads to partial wilting. As the disease progress the upper crown region and senescence. Finally, infection spreads to the whole plant resulting in mortality of plants. Brown discoloration of xylem vessels with slight variation in symptomatology depending on severity of infection is also observed [4].

It draws an attention of the present study to know the hot spots for *Ceratocystis* wilt in Karnataka. Hence, a preliminary rowing survey in Karnataka was the need of an hour to assess the wilt incidence and yield loss due to pathogen**.** As a result, various fungicides, bioagents and botanicals were tested against the *C.fimbriata* under *in vitro* conditions, which are crucial components of the integrated disease management. with respect to locality. The different fungicides, bioagents and botanicals are critical components of plant disease management [10].

2. MATERIALS AND METHODS

2.1 Survey for Occurrence of Pomegranate Wilt Disease Incidence in Southern Karnataka

The survey was carried out during 2019-20 to 2020-21 to know the incidence of wilt disease of pomegranate in Ramanagara, Bangalore Rural,

Chitradurga, Chikkaballapur and Tumakuru districts. The survey was conducted in places like Maralavadi, Hosadurga, Mullahalli and Gerehalli (Ramanagara district), Veerapura and Maddur (Bangalore Rural district), Yelladakere, Marikanive and Chigalakatte (Chitradurga district), Manchenhalli, Gudibanada, Mandalahalli and Bagepalli (Chikkaballapur district), Sira and Chiknayakanhalli (Tumakuru district).

The number of wilted plants was counted and Percent disease incidence was calculated. The complete wilted plants were collected for isolation and other studies. The percent disease incidence was calculated using following formula.

$$\text{Per cent Disease Incidence} = \frac{\text{Number of plants infected}}{\text{Total number of plants observed}} \times 100$$

2.2 Collection and Isolation of the Pathogen

The standard tissue isolation technique was followed for isolation of wilt pathogen from infected stem collected during survey. Two mm size of infected stem parts were surface sterilized with 0.1 per cent mercury chloride and washed in with sterile water thrice to remove traces of mercury chloride. The bits were placed on potato dextrose agar and allowed for the growth of the fungus. The pathogen confirmed based on morphological characters.

2.2.1 Purification of isolated culture

Hyphal tip isolation was done on water plates. Dilute spore suspension of the pathogen was prepared in sterilized distilled water containing eight to ten spores per ml from 15 days old culture. One ml of such suspension was spread uniformly on two per cent solidified water agar plates and observed for spores under the microscope. Single spore was marked with a marker on backside of the Petri plate and it was allowed to germinate. Such plates were periodically observed for spore germination under microscope. The hyphae growing from each cell of the single spore was traced and marked with marker. The tip of the hyphae was cut carefully and transferred to PDA plates and incubated at 25 ± 2°C for 15 days. Later, mycelial bits of the fungus were transferred in the centre of petri plates containing PDA and incubated at 25 ± 2°C for 15 days. Saltation or sectoring was observed in the culture to confirm the pure culture of the fungus.

2.3 Identification of the Fungus

The fungus was identified based on morphological characteristics such as type of mycelium, fruiting body and hat shaped ascospores by using microscope.

2.4 Molecular Identification, Sequencing of 18S rDNA Region and *in silico* Analysis

The mycelium of fungus collected from the potato dextrose broth after 7 days of incubation was filtered by using Whatman No.40 filter paper. The mycelia were later dried by pressing in between folds of pre-autoclaved filter papers. The DNA extraction of fungus was carried out by following CTAB method.

The 18S rDNA region was sequenced to confirm the identity of organism. The PCR product was sequenced in both the directions using Sanger di-de-oxy method at Europhins, Bengaluru. Homology search done using BLAST algorithm available at the VA3T.ncbi.nlm.nih.gov. Multiple alignments for homology search performed using the Clustul W algorithm software and the phylogenetic tree was constructed.

2.5 Cultural Characters of *C. fimbriata* on Different Solid Media

Twenty ml of each sterilized and cooled medium *viz.,* Potato dextrose agar, V8 juice agar, Potato Carrot agar, Czapeck's agar, Richards agar, Oat meal agar, Corn meal agar, Rose Bengal agar, Malt extract agar, Sabouraud dextrose agar, Water agar and Nutrient agar were poured aseptically and separately into sterilized Petri plates. Five mm disc of the *C. fimbriata* was taken from actively growing culture with the aid of cork borer and a disc was placed at the centre of Petri dish and then incubated at 25 ± 1°C for 12 days. Each of this experiment was replicated thrice and observations regarding cultural characteristics such as the color, diameter, and pigmentation of the colony were recorded.

2.6 Physiological Studies

2.6.1 Effect of temperature and pH on the growth of *C. fimbriata*

The fungal growth was tested at 15, 20, 25, 30 35 and 40°C. For each treatment, three replications were maintained. This study was

carryout on both solid and liquid media. The mycelial mat in PDB was harvested by filtering through Whatsman No.1 filter paper of 9 cm diameter and dried. The dry mycelial weight was recorded and also diameter of plates was measured and results were analyzed statistically.

For pH studies PDB, liquid media was adjusted to required pH *viz.,* 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, and 8.5, the ideal pH for growth of the fungus was determined by harvesting mycelial mat that was filtered through Whatsman filter paper. The flasks (effect of pH) and plates (effect of temperature) were inoculated with 5 mm disc of fungus which was collected from 12 days old culture and incubated at respective temperatures. The mycelial mat in PDB was harvested by filtering through Whatsman No.1 filter paper of 9 cm diameter and dried. The dry mycelial weight was recorded and also diameter of plates was measured and results were analyzed statistically.

2.7 Evaluation of Different Fungicides, Botanicals and Bio-Agents Against *C. fimbriata*

2.7.1 *In vitro* evaluation of fungicides

The effectiveness of 19 fungicides, including six non-systemic, six systemic, and seven combi-products, was evaluated against *C. fimbriata* using the poisoned food technique. Each fungicide was tested at different concentrations (250 ppm, 500 ppm, and 1000 ppm). The efficacy of six non-systemic (Chlorothalonil, Copper oxy chloride, Captan , Zineb, Mancozeb and Propineb: four different concentration 100, 250, 500 and 1000 ppm), six systemic fungicides (Acrobat, Thiophenate methyl, Tebuconozole, Difenconozole, Carbendazim and Propiconozoleat: four different concentration 100, 250, 500 and 10 ppm) and seven combi-products (Tricyclazole 18% + Mancozeb 62%WP, Iprovalicarb5.5% + Propineb 61.25%WP, Metalaxyl 8%WP + Mancozeb 64% , Mancozeb 63%WP + Carbendazim 12%, Tricyclazole 45% WG+Hexaconazole10% , Hexaconazole 5% WP + Captan 70% : four different concentration 100, 250, 500 and 1000 ppm). The efficacy of different fungicides was expressed as Percent inhibition of mycelial growth over control was calculated by using the formula suggested by Vincent [11].

$$I = \frac{C - T}{C} \times 100$$

Where

I = Per cent inhibition of mycelial growth
C = Growth of mycelium in control.
T = Growth of mycelium in treatment

2.7.2 *In vitro* evaluation of bio-agents against *C. fimbriata*

Seven fungal bio-agents [(*Trichoderma viride* (Tv-2), *T. harzianum* (Th-14), *T. viride* (Tv-1), *T. harzianum* (Th-41), *T. harzianum* (Th-55), *T. harzianum,* (Th-56), and *T. viride* (Tv-3)] and bacterial bio-agents [*Bacillus subtilis* (Dharwad), *B. megatherium* (Gkvk), *B. subtilis* (Gkvk), *Pseudomonas fluorescence* (Chintamani), *P. fluorescence* (Gkvk), *B. megatherium* (Dharwad), and *P. fluorescence* (Dharwad)] were tested against *C. fimbriata* by dual culture technique. Percent inhibition is calculated by using the formula suggested by Vincent [11].

2.7.3 *In vitro* evaluation of botanicals against *C. fimbriata*

The efficiency of plant extract or botanicals were tested against *C. fimbriata* on PDA medium by using poisoned food technique. For this, fresh plant parts (leaves/bulb) of 100 g of each as mentioned below were collected, washed with tap water and then distilled water. The fresh sample was chopped and crushed by adding sterile water of 100 ml. The crushed product was filtered through muslin cloth. The filtrate solution gave 100 per cent and which was used as stock solution. Five, ten, fifteen and twenty ml of stock solution was mixed with 95, 90, 85 and 80ml of PDA medium and then it was shaken for uniform mixing of plant extract. Later, the media was sterilized and allowed it to cool. Twenty ml of medium was poured into sterilized Petri plates and then fungal disc of five mm was placed at the center of Petri plate and then such plates were incubated at 25 ± 1°C. The control plate was maintained on PDA medium without any plant extract. The radial growth of fungus was recorded in treatment plates when colony growth reached periphery in control plate. The per cent inhibition of mycelial growth of test fungus was calculated by using the formula suggested by Vincent [11].

3. RESULTS AND DISCUSSION

3.1 Symptoms, Isolation and Identification

In field condition, symptoms initiated as yellowing of leaves of one or more branches and the plant

appeared devitalized, leaves turned pale yellow starting from lower branches/limbs and progressed upwards. The partial wilting of the tree with drying and death of some branches were common symptoms (Figs. 1, 2 and 3). Standard tissue isolation was followed to isolate *C. fimbriata* from diseased sample of infected root showing typical symptom of dark grayish brown streaks on splitting of root portion, collected from pomegranate field. Isolation of the pathogen was done on potato dextrose agar by placing d. Conidiophore producing cylindrical endoconidia. infected bits on the media. (Figs. 1, 2, and 3).

Light greyish colour mycelial growth was observed within 3-4 days after inoculation. Further, the isolated fungus was purified by hyphal tip isolation method and the culture was maintained at 28 ± 2°C on PDA (Fig. 4).

3.2 Survey for the Incidence of wilt of Pomegranate in Karnataka

A roving survey was conducted to know the intensity of wilt disease caused by *C. fimbriata* in major pomegranate growing regions of Karnataka during 2020. The disease incidence was observed in all locations surveyed and it ranged from 1.14 to 62.06 per cent (Table 1). Among surveyed locations, maximum wilt incidence was observed in Yelladakere (62.06%) of chitradurga district and least incidence in Chigalakatte (1.14 %) of Chitradurga district. Maralavadi (52.00); Hosadurga (29.2); Mullahalli (15.20); gerehalli (8.96); Veerapura (40.00); Maddur (5.00); Yelladakere (62.06);Marikanive (5.00);Chigalakatte (1.14);Manchenhalli (14.23); Gudibande (12.96); Mandalahalli (5.69)Bagepalli (2.4);Sira (11.60); Huliyar 18.44); Kaladagi (12.00);Indi (15.00); vijayapur (12.00).

3.3 Molecular Confirmation of the Pathogen *C. fimbriata*

Species identification and confirmation of *C. fimbriata* by using morphological characters is a complex and challenging process that requires other identification techniques. Molecular technique can be employed in addition to morphological characters for identification and confirmation of *C. fimbriata.*

The DNA from wilt causing fungus was isolated and amplified through PCR using universal ITS primers *viz*., ITS1 and ITS4. The amplified product was later subjected to gel electrophoresis in 1.5 % agarose gel. The amplicon size of 650 bp was obtained (Fig. 3).

“The homology search was done by using the bioinformatics tool NCBI (National Centre for Bioinformatics) BLAST program. The amplicon sequence has shown 100 to 78 percent similarity with the existing *C.fimbriata* in NCBI Genbank through BLAST analysis and was assigned with accession number” (OK597212). It confirmed the identity of *C.fimbriata* causing wilt in pomegranate. For the construction of a phylogenetic tree, *C. punicae* was used as an outgroup to interpret the clustering of isolate *C. fimbriata* causing wilt in pomegranate. The phylogram this isolate revealed that there are two major clusters, cluster A comprises Latin American isolates, our isolate also lie under this group with the isolate name of Maralavadi isolate, having homology of 85 per cent with cluster B, However, the reference isolate *C. punicae* formed a distinctive, separate clade (Fig. 5). “Phylogenetic analyses placed the pomegranate isolates from China among members of the LAC of the *C. fimbriata* complex; specifically, to populations that appear to be native to eastern and northern South America” [12]. “The genus *Ceratocystis* contains a number of emerging plant pathogens, mostly members of the Latin American Clade (LAC), in which there are several unresolved taxonomic controversies. Among the most important are Brazilian pathogens in the *C. fimbriata* complex, *C. manginecans* and *C. eucalypticola*. Representatives of *C. manginecans* and *C. eucalypticola* from India and China, respectively, were shown to be fully interfertile in laboratory matings and hybrids between the putative species were identified on *Punica* in India. An Indian tester strain was sexually compatible with representatives of what has been considered *C. fimbriata* on numerous hosts across Brazil. An expanded concept of *Ceratocystis manginecans* and five new species in the Latin American Clade of Ceratocystis” [13].

3.4 Cultural Studies of *C. fimbriata* Growth on Different Solid Media

The results obtained on the cultural characters of the pathogen using twelve different solid media is presented in Table 2, Plate 6 and Fig 3. The colony growth of the fungus was significantly superior and faster on Potato dextrose agar medium with colony diameter of 90.00 Potato carrot agar and corn meal agar were on par to each other with colony diameter of 75.67 mm

Table 1. Survey for severity of wilt disease on pomegranate in southern Karnataka

Sl. No.	District	Taluk	Village/ town	Per cent incidence of Wilt
1	Ramanagara	Kanakapura	Maralavadi	52.00
		Channapatna	Hosadurga	29.12
			Mullahalli	15.20
			Gerehalli	8.96
	Mean	**26.32**		
2	Bangalore Rural	Doddaballapur	Veerapura	40.00
		Nelamangala	Maddur	5.00
	Mean	**22.50**		
3	Chitradurga	Hiriyuru	Yelladakere	62.06
			Marikanive	5.00
			Chigalakatte	1.14
	Mean	**22.73**		
4	Chikkaballapur	Gauribidanur	Manchenhalli	14.23
		Gudibanda	Gudibande	12.96
			Mandalahalli	5.69
		Bagepalli	Bagepalli	2.40
	Mean	**8.82**		
5	Tumakuru	Sira	Sira	11.60
		Chiknayakanhalli	Huliyar	18.44
	Mean	**15.02**		

Table 2. Effect of different cultural media on the growth of *C. fimbriata*

Sl. No.	Different media	Radial growth(mm)	Colony colour	Type of margin	Colony Texture	Growth nature	Sporulation
1	Potato dextrose agar	90.00	Dark grey	Regular	Smooth	Flat	+
2	Potato carrot agar	75.67	Light grey	Regular	Smooth	Flat	+
3	Corn meal agar	75.12	Dark grey	Regular	Smooth	Flat	+
4	Malt extract agar	67.133	Light grey	Regular	Smooth	Flat	+
5	Oat meal agar	62.17	Greyish whiten	Regular	Cottony	Raised	+
6	Rose Bengal agar	46.27	Light grey	Irregular	Smooth	Raised	+
7	Sabouraud dextrose agar	46.00	Light grey	Irregular	Smooth	Raised	+

Sl. No.	Different media	Radial growth(mm)	Colony colour	Type of margin	Colony Texture	Growth nature	Sporulation
8	V-8 juice agar	42.17	Dark grey	Irregular	Smooth	Flat	+
9	Richard's agar	33.47	Light brown	Regular	Smooth	Flat	+
10	Czapek's Dox agar	24.10	Light brown	Regular	Smooth	Flat	+
11	Nutrient agar	13.00	Light grey	Regular	Smooth	Flat	-
12	Water agar	12.17	Light grey	Regular	Smooth	Flat	-
S. Em ±		0.45					
CD @ 1%		1.78					

Fig. 1. Different types of wilt

Fig. 2. Severely infected pomegranate wilt plot

Fig. 3. Different kind of symptoms on stem

Fig. 1, 2 and 3. Different types of wilt symptoms, severely infected pomegranate wilt plot and different kind of symptoms on stem

and 75.12mm respectively. However, the least growth was recorded on water agar and nutrient agar media and were on par with each other with colony diameter of 12.17 mm and 13.00 mm respectively.

During the study, variation in colony characters of the pathogen was observed on different solid media with respect to colony colour, type of margin, colony texture and growth nature and sporulation (Table 2). The colony colour varied from greyish white to dark grey (Fig. 6).

Colony texture varied from smooth to cottony colony. All the tested media recorded smooth colony growth except oat meal agar with cottony growth. Sporulation was observed on all the tested media except nutrient and water agar. The variation in the growth and sporulation of the pathogen was probably due to the nutrient source present in the media therefore, some media supported the growth very well but others fail to support good growth and sporulation. The results are supported by the findings of Brito et al. [14] who reported that the PDA and V8-agar media showed the highest mycelial growth. Gururaj et al. [15] who reported that the growth of *C. fimbriata* on different solid media was raised and mycelial colour was white to greyish and colony margin in Petri plate was regular to irregular.

3.5 Effect of Temperature Levels on Growth of *C. fimbriata* on Solid Media and Liquid Media

Temperature is the most important factor that, influencing the growth and metabolism of *C. fimbriata*. Different levels of temperature *viz.*, 15°C, 20°C, 25°C, 30°C, 35°C and 40°Cwere studied.

The results obtained on the effect of different temperature levels on solid and liquid media indicated that the growth and morphological characters of the fungus varies with the varying temperature. Pathogen can survive under wide range of temperature regimes but minimum, optimum and maximum temperature are required for their growth.

The variation in the growth of the pathogen among the different temperature levels on different solid media was found statistically significant. (Table 3 and Fig. 7). Among the different temperature levels evaluated, 30°C was found to be the best temperature for growth of the pathogen and the next best was 25°C. At 30°C the growth was supported very well and the complete growth of 90.00 mm diameter was observed. At 25°C the colony diameter of 55.55 mm was observed. The colony diameter of 31.00 and 26.83 mm was recorded at 35°C and 20°C respectively. The growth of the pathogen was not observed at 15°C and 40°C. The results are in conformity with the findings of Yadahalli (2005), who reported that *C. paradoxa* grew well and sporulated well in the temperature range of 25-28°C.

Different levels of temperature *viz.*, 15°C, 20°C, 25°C, 30°C, 35°C and 40°C were studied. The variation in the growth of the pathogen among the different temperature levels on different solid media was found statistically significant. (Table 3 and Fig. 8). Among the different temperature levels evaluated, 30°C was found to be the best temperature and growth of the pathogen was not observed at 15°C and 40°C.

3.6 Effect of Hydrogen Ion Concentration (pH) on the Growth of *C. fimbriata* on Liquid Media

The hydrogen ion concentration influences the growth of pathogen. Every organism has its own maximum, optimum and minimum pH levels for its growth and development. The results of the pH requirement of *C. fimbriata* for its growth was studied on six different media and the data pertaining to that is presented in the Table 4.

The growth of the pathogen was supported at all the different pH levels tested. Different levels of pH *viz.*, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9 and 9.5 were studied. The maximum growth of the pathogen was recorded at pH 7 with dry mycelial weight of 0.278 mg and was found on par with pH 6.5, the least mycelial weight was observed at pH 4 with dry mycelial weight of 0.014 mg. The present study is supported by the findings of Rehaman et al. [16] reported that the colony growth of *C. maginecans* on different pH levels, the colony growth was maximum at pH level 7 followed by 8.

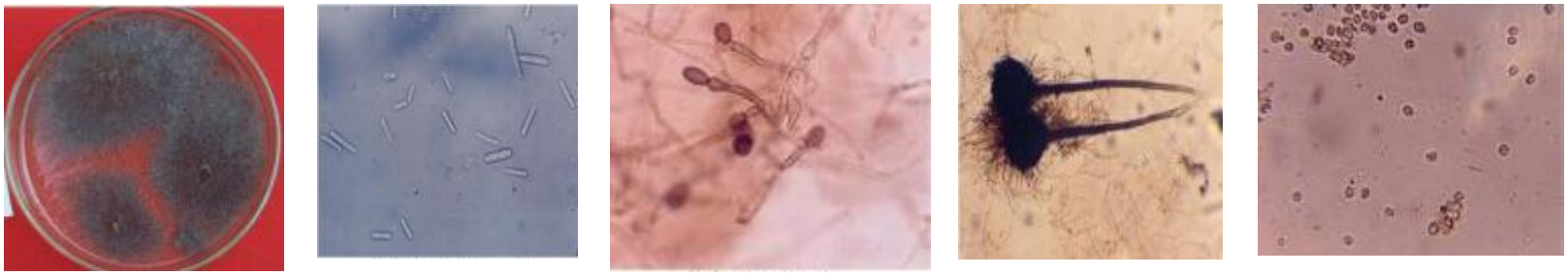
3.7 *In vitro* Evaluation of Fungicides, Bio Agents and Botanicals Against *C. fimbriata*.

3.7.1 *In vitro* evaluation of systemic fungicides against *C. fimbriata*

Six systemic fungicides were tested at four concentrations *viz.,* 100, 250, 500 and 10 ppm

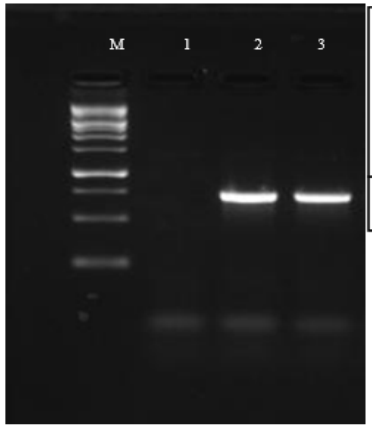
Table 3. Effect of temperature on growth of *C. fimbriata*

Sl. No.	Temperature (°C)	Potato dextrose broth Dry mycelial weight (mg)	Potato dextrose agar Radial growth (mm)
1	15	0.00	0.00
2	20	0.46	26.83
3	25	0.63	55.50
4	30	0.85	90.00
5	35	0.53	31.00
6	40	0.00	0.00
SEm ±		0.02	0.40
CD @ 1%		0.10	1.73



A. Colony of *C. fimbriata* | **B. Cylindrical endoconidia** | **C. Aleurioconidia** | **D. Perithecium** | **E. Hat shaped ascospores**

Fig. 4. Morphology of pomegranate wilt pathogen *Ceratocystis fimbriata*



M-1kb Ladder

Lane 1: Negative control

Lane 2, 3: *Ceratocystis fimbriata* isolate

~ 650bp

Fig. 5a. PCR amplification of 18s r DNA fragment from sample. The size of PCR amplified product is ~ 650bp

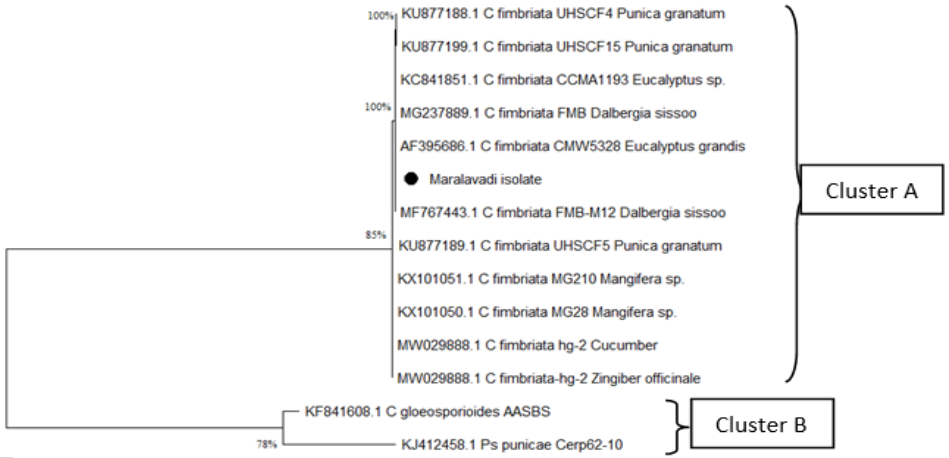


Fig. 5b. Phylogenic tree construction using MegaX software

Table 4. Effect of pH on the growth of *C. fimbriata* in liquid media

Sl. No.	pH level	Dry mycelial weight (g)
1	4.0	0.014
2	4.5	0.039
3	5.0	0.078
4	5.5	0.108
5	6.0	0.226
6	6.5	0.238
7	7.0	0.278
8	7.5	0.222
9	8.0	0.174
10	8.5	0.129
11	9.0	0.088
12	9.5	0.027
SEm ±		**0.03**
CD @ 1%		**0.10**

Table 5. *In vitro* evaluation of systemic fungicides against *C. fimbriata*

Sl. No.	Systemic fungicides	Percent inhibition				
		Concentration (ppm)				
		100	**250**	**500**	**1000**	**Mean**
1	Dimethomorph	22.22	29.88	58.89	90	50.23
2	Thiophanate methyl	21.67	80.56	86.11	10	72.08
3	Difenconazole	27.78	82.59	91.11	100	75.37
4	Tebuconazole	28.33	84.44	90.74	100	75.88
5	Carbendazim	36.11	83.89	100	100	80.00
6	Propiconazole	38.33	86.67	100	100	81.25
Mean		29.07	74.661	87.80	98.33	
		Fungicides (F)	Concentration (C)		Interaction (F×C)	
SEm ±		0.44	0.36		0.89	
CD @ 1%		1.28	1.04		2.56	

Table 6. *In vitro* evaluation of non - systemic fungicides against *C. fimbriata*

Sl. No.	Non-systemic fungicides	Percent inhibition over control				
		Concentration (ppm)				
		100	**250**	**500**	**1000**	**Mean**
1	Copper oxychloride	52.04	54.07	57.78	66.67	57.67
2	Chlorathanil	60.56	61.67	66.11	68.89	64.31
3	Zineb	38.33	51.85	72.59	100.00	65.69
4	Captan	52.96	77.77	95.00	100.00	81.43
5	Mancozeb	69.44	100.00	100.00	100.00	92.36
6	Propineb	71.67	100.00	100.00	100.00	92.98
Mean		56.38	73.88	81.17	89.26	
		Fungicides(F)	Concentration (C)		Interaction (F x C)	
SEm ±		0.22	0.18		0.44	
CD @ 1%		0.64	0.52		1.28	

against *C. fimbriata* under lab conditions by using poison food technique. The percent inhibition of mycelial growth of *C. fimbriate* in different systemic fungicides on solid media is presented in (Table 5, and Fig. 9). Among the systemic fungicides tested, propiconazole and carbendazim, were significantly superior and on par with mean mycelial inhibition of 81.25 and 80.00% respectively. Out of the four concentrations, 500 and 1000 ppm was found to

be most effective with percent inhibition. However, dimethomorph was least effective with mean mycelial inhibition of 50.23 per cent.

The results are supported by the findings of Sharma and Khosla (2020), reported that the propiconazole, carbendazim and hexaconazole gave cent % inhibition of test fungus *C. fimbriata.*

3.7.2 *In vitro* evaluation of non-systemic fungicides against *C. fimbriata*

The results presented in Table 6 revealed that, there is significant difference between different contact fungicides in Percent mycelial inhibition. Among the contact fungicides tested, propineb and mancozeb were found to be best and on par to each other with mean mycelial inhibition of 92.98 and 92.36 % and the least inhibition was observed in copper oxy chloride at all the different concentration levels tested, with mean mycelial inhibition of 54.17 per cent. However, inhibition of 66.68, 53.33, 51.67 and 45.00 % was recorded at 1000, 500, 250 and 100 ppm respectively (Fig. 10).

The obtained results were in agreement with the findings of Sharma and Khosla [17], who reported that the contact fungicides mancozeb and propineb were most effective with cent %inhibition of the pathogen *C. fimbriata.* The similar results were reported by Raja (2017), that the non systemic fungicides captan, mancozeb and zineb recorded maximum inhibition of mycelial growth at all concentrations (0.10%, 0.20% and 0.30%).

3.7.3 *In vitro* evaluation of combi fungicides against *C. fimbriata*

Six combi-product fungicides were tested at four concentrations *viz.,* 100, 250, 500 and 1000 ppm by using poisoned food technique under *in vitro* condition. The % inhibition of mycelial growth of *C. fimbriata* in different combi fungicides is presented in (Table 7 and Fig. 11).

Out of six combi fungicides tested carbendazim 12% + mancozeb 63% was most effective and superior over other fungicides with complete inhibition of the fungus. Next in the order was hexaconazole 5% + captan 70% WP with mean mycelial inhibition of 96.39 per cent. With respect to interactions complete inhibition was observed at 1000 and 500 ppm however, 93.33 and 92.22% inhibition was observed at 250 and 100 ppm respectively. Tricyclazole 18% + mancozeb 62% WP with mean mycelial inhibition of 80.14 and with respect to the interactions in complete inhibition was observed in 1000 ppm however, mycelial inhibition of 87.22, 85.00 and 48.33 %was observed at 500, 250 and 100 ppm. Next in tricyclazole 45% + hexaconazole 10% WG with mean mycelial inhibition of 79.12 %and with respect to complete inhibition was recorded at 1000 and 500 ppm however, 92.59, 23.89 %inhibition was observed at 250 and 100 ppm respectively. Next in the order was metalaxyl 8% + mancozeb 64% WP with mean mycelial inhibition of 59.77 %and recorded complete inhibition at 1000ppm however, 57.79, 43.33 and 42.96 %mycelial inhibition was observed at 500, 250 and 100 ppm respectively. The least mycelial inhibition was observed in Iprovalicarb 5.5% + propineb 61.25% WP with 42.64 %and found least effective in all the tested concentrations with mycelial inhibition of 60.00, 50.55, 31.11 and 28.89 %at 1000, 500, 250 and 100 ppm respectively.

The maximum inhibition of the pathogen by combi fungicides was may be due to the combined and synergistic effects of both contact and systemic fungicides against the pathogen. The results depicted that the combination of carbendazim 12% + mancozeb 63% was most effective and superior over other fungicides with complete inhibition of the fungus. The results are supported by the findings of Sharma and Khosla [17] who reported that the combination of fungicides carbendazim 12% + mancozeb 63% showed cent % inhibition of the test fungus followed by captan 70%+ hexaconazole 5%.

3.7.4 *In vitro* evaluation of fungal bio-agents against *C. fimbriata*

The antagonistic action of selected seven fungal biocontrol agents against C. fimbriata was carried out through dual culture technique. Based on the radial growth of the bio-agent and fungus, % inhibition was calculated. The results are presented in the Table 8, and Fig 12. Among the fungal bio-agents tested, *T. viride* (Tv-3) was found to be most effective and significant over other biocontrol agents with maximum mycelial inhibition of 80.55% over control. Similar inhibition of the pathogen was observed in *T. harzianum* (Th-55) and *T. harzianum* (Th-56) with mean mycelial inhibition of 75.00 per cent. Next in the order was *T. harzianum* (Th-41) with 73.88 % inhibition followed by *T. viride* (Tv-1) with 63.14 % of mycelial inhibition and *T. harzianum* (Th-14) with mycelial inhibition of 45.74 per cent.

Table 7. Evaluation of combi product fungicides against *Ceratocystis fimbriata*

Sl. No.	Combi products	Percent inhibition over control				
		Concentration (ppm)				
		100	250	500	1000	Mean
1	Iprovalicarb 5.5% + Propineb 61.25%	28.89	31.11	50.55	60.00	42.64
2	Metalaxyl 8%WP+ Mancozeb 64%	42.96	43.33	52.79	100.00	59.77
3	Tricyclazole45%WG + Hexaconazole 10%	23.89	92.59	100.00	100.00	79.12
4	Tricyclazole 18%+ Mancozeb 62%WP	48.33	85.00	87.22	100.00	80.14
5	Hexaconazole 5%WP+ Captan 70%	92.22	93.33	100.00	100.00	96.39
6	Mancozeb 63%WP + Carbendazim 12%	100	100	100	100	100.00
Mean		56.049	74.228	81.759	93.333	
S.Em ±	Fungicides (F) 0.31		Concentration (C) 0.25		Interaction (F×C) 0.61	
CD @ 1% 0.88			0.72		1.75	

Table 8. *In vitro* evaluation of fungal bio-agents against *C. fimbriata*

Sl. No.	Fungal bio-agents	Isolate	Percent inhibition over control
1	*Trichoderma viride*	(Tv-2)	43.14
2	*Trichoderma harzianum*	(Th-14)	45.74
3	*Trichoderma viride*	(Tv-1)	63.14
4	*Trichoderma harzianum*	(Th-41)	73.88
5	*Trichoderma harzianum*	(Th-55)	75.00
6	*Trichoderma harzianum*	(Th-56)	75.00
7	*Trichoderma viride*	(Tv-3)	80.55
S Em±			0.25
CD @ 1%			1.05

Table 9. *In vitro* evaluation of bacterial bio-agents against *C. fimbriata*

Sl. No.	Bacterial bio-agents	Isolate	Per cent inhibition over control
1	*Bacillus subtilis*	Dharwad	63.33
2	*B. megatherium*	Gkvk	54.63
3	*B. subtilis*	Gkvk	52.78
4	*Pseudomonas fluorescence*	Chintamani	49.26
5	*P. fluorescence*	Gkvk	39.81
6	*B. megatherium*	Dharwad	35.74
7	*P. fluorescence*	Dharwad	30.00
SEm±			0.42
CD @ 1%			1.76

The least mycelial inhibition was observed in *T. viride* (Tv-2) with 43.14%. The inhibitory effect of these fungal bio-agents probably due to hyperparasitism, competition for space and nutrients or antibiosis.

The findings are in collaboration to the earlier study conducted by Kadam et al. [18] who reported that the *T. viride* recorded the significantly highest mycelial growth inhibition of 86.85 %of the test pathogen *C. fimbriata*. The similar results were observed by the findings of Somasekhara [19] who reported that the bio-agents *T. viride* and *T. harzianum* were effective against the pathogen.

3.7.5 *In vitro* evaluation of bacterial bio-agents against *C. fimbriata*

The antagonistic actions of selected seven bacterial biocontrol agents *C. fimbriata* was tested through dual culture technique. Based on

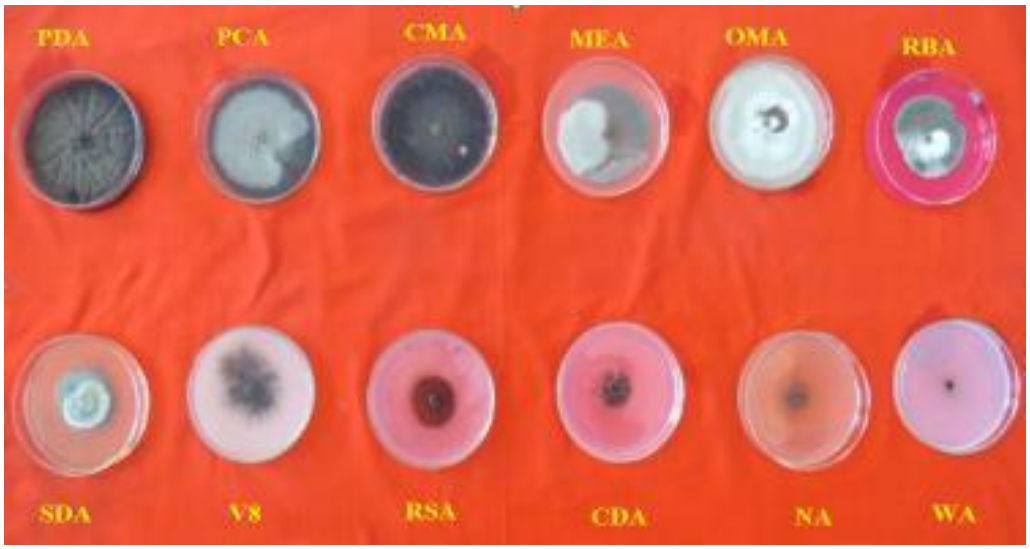


Fig. 6. Growth of *C. fimbriata* on different cultural media

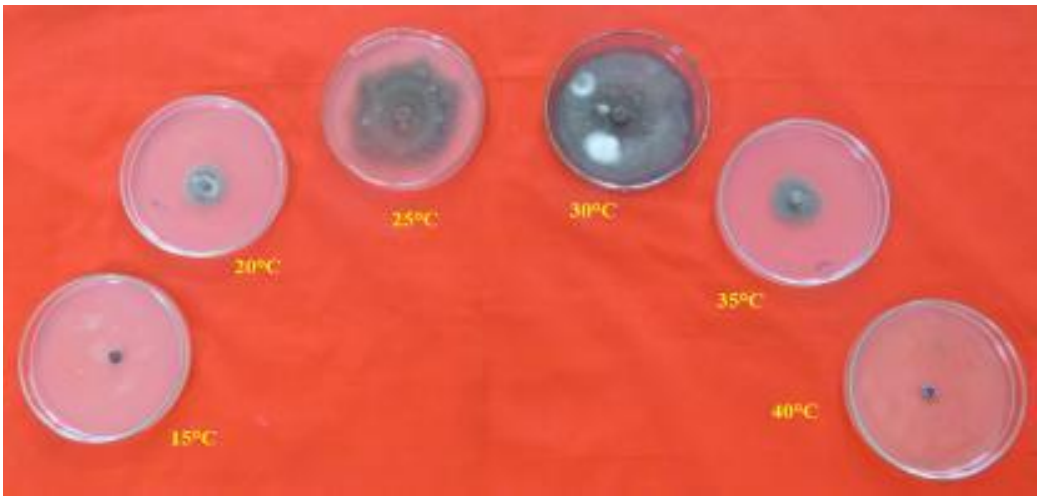


Fig. 7. Effect of temperature on the growth of C. fimbriataon potato dextrose agar

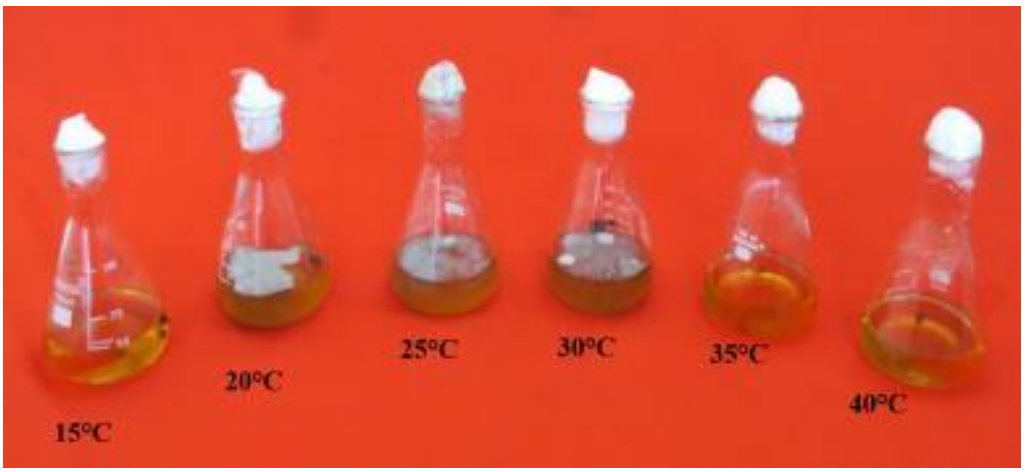


Fig. 8. Effect of temperature on the growth of C. fimbriataon potato dextros broth

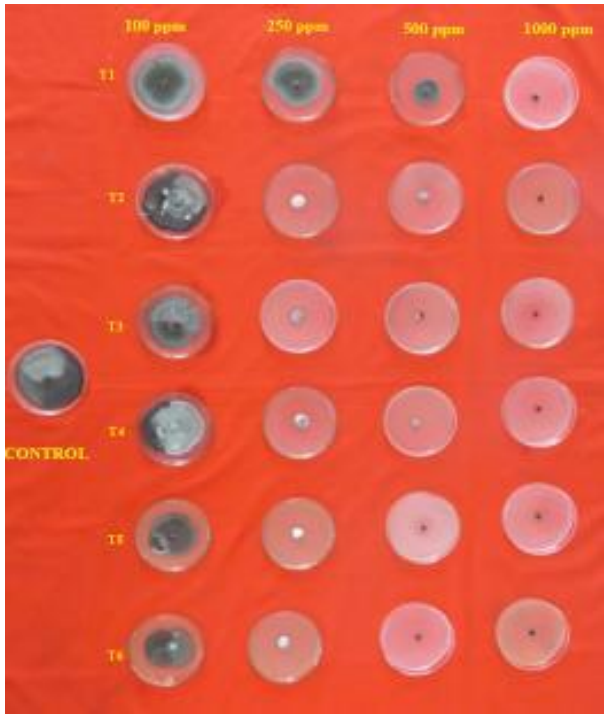


Fig. 9. Systemic fungicides against *C. fimbriata*
T1.Acrobat T2.Thiophenate methyl T3. Tebuconozole T4.Difenconozole T5. Carbendazim T6.Propiconozole

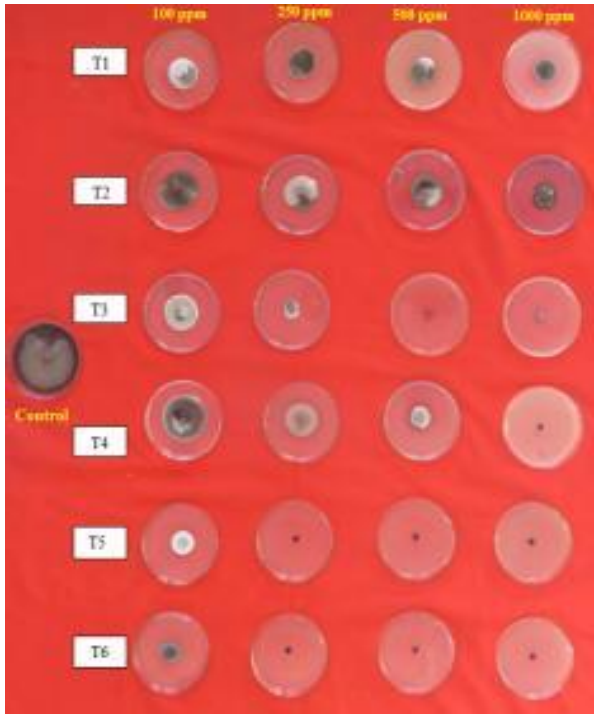


Fig. 10. Non-systemic fungicides against *C. fimbriata*
T1. Chlorothalonil T2. Copper oxy chloride3.Captan T4. ZinebT5.Mancozeb T6. Propineb

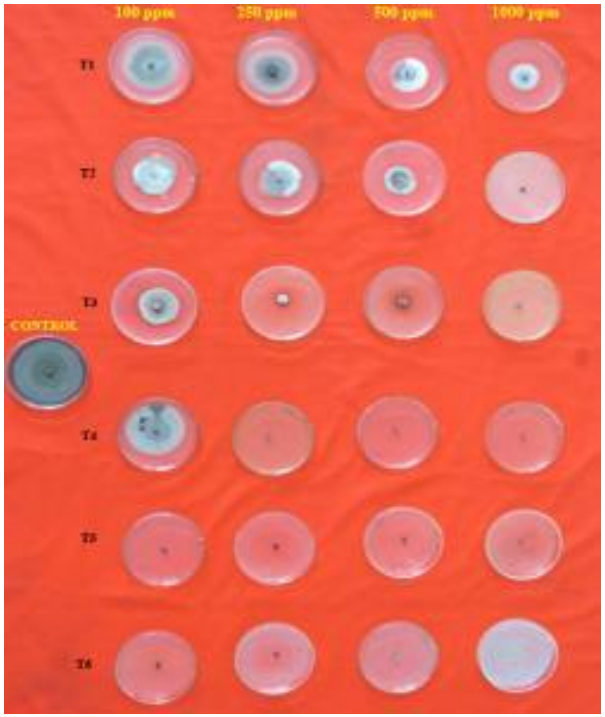


Fig. 11. Combi-product fungicides against *C. fimbriata*
T1. Tricyclazole +Mancozeb T2.Iprovalicarb + Propineb T3. Metalaxyl + Mancozeb T4.Mancozeb + Carbendazim T5. Tricyclazole +Hexaconazole T6. Hexaconazole + Captan

Fig. 12. *In vitro* evaluation of fungal bio-agents against *C. Fimbriata*

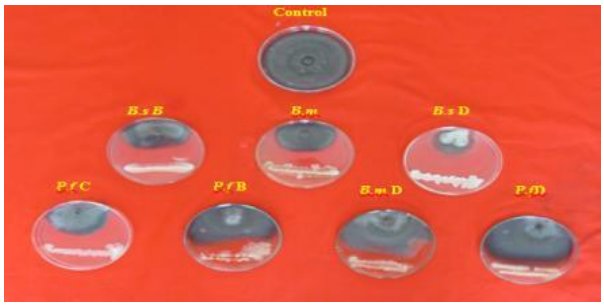


Fig. 13. *In vitro* evaluation of bacterial bio-agen against *C.fimbriata*

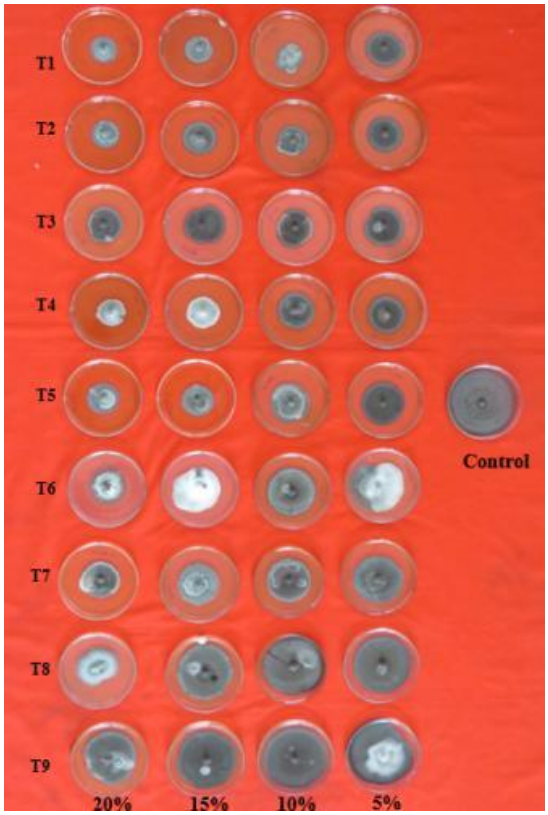


Fig. 14. *In vitro* evaluation of botanicals against C. fimbriata

T1. Pongamia T2. Subabul T3. Garlic T4. Simaroba
T5. Tulsi T6. Onion T7. Lemongrass
T8.Neem T9. Ginger

the observation of radial growth of the bio-agent and fungus, the %inhibition was calculated. The results are presented in the Table 9, and Fig 13. Among the tested bacterial bio-agents, *B. subtilis* (Dharwad) was significantly superior over control with 50.70% mycelial inhibition followed by *B. megatherium* (49.81%), *B. subtilis* (GKVK) with 47.03% mycelial inhibition. Next in the order was *P. fluorescence* (Chintamani) with mycelial inhibition of 40.00% followed by *P. fluorescence* (GKVK) with 39.81% and *B. subtilis* -1 with 35.74% mycelial inhibition. The least inhibition was recorded in *P. fluorescence* (Dharwad) with 30%.

The inhibitory effect of the bio agents may be due to competition for space and nutrients, production of volatile compounds and antibiosis. The findings are in collaboration with the study conducted by Khan *et al.* [20] who reported that the *Pseudomonas fluorescens* was effective in

Table 10. *In vitro* evaluation of botanicals against *C. fimbriata*

Sl. No.	Botanicals	Percent inhibition over control				
		Concentration				
		5 %	10%	15%	20%	Mean
1	Pongamia (Leaves)	52.19	60.8	62.74	63.58	59.83
2	Simarouba(Leaves)	47.78	57.67	62.59	64.26	58.07
3	Subabul(Leaves)	48.11	49.25	54.52	57.28	52.29
4	Garlic (Bulb)	38.85	48.62	52.63	55.68	48.94
5	Lemongrass(Leaves	29.44	40.74	43.70	45.00	39.72
6	Tulsi(Leaves)	28.52	33.33	42.11	42.80	36.69
7	Onion (Bulb)	28.89	33.41	42.22	42.07	36.65
8	Ginger (rhizome)	14.68	19.99	30.37	46.04	27.79
9	Neem(Leaves)	20.15	20.85	23.33	45.74	27.52
mean		34.28	40.53	46.02	51.38	
		Botanicals (F)	Concentration (C)		Interaction (F×C)	
SEm ±		0.106	0.071		0.212	
CD @ 1%		0.299	0.199		0.598	

controlling the growth of the pathogen among all the bacterial bio-agents tested. The results are also supported by the findings of Apet et al. [21] who reported that the *P. fluorescens* was effective in inhibiting the growth of the pathogen *C. paradoxa* with mycelial inhibition of 67.36 per cent.

3.7.6 *In vitro* evaluation of botanicals against *C. fimbriata*

The antagonistic actions of selected nine botanicals were tested against *C. fimbriata* by using poison food technique. Based on the observation of radial growth of the fungus, the % inhibition was calculated. The results are presented in the Table 10, and Fig 14.

Among the tested botanicals the maximum mycelial inhibition of 59.83 %was observed in pongamia followed by simarouba (58.07%), subabul (52.29%), garlic (48.94%) and lemon grass (39.72%). Leaf extract of tulsi and bulb extract of onion were found on par with each other with mycelial inhibition of 36.69 and 36.65 per cent. Therhizome extract of ginger and leaf extract of neem were found on par with each other with 27.79 and 27.52 % respectively. However, the leaf extract of neem was least effective in controlling the mycelial growth of the pathogen [22].

4. CONCLUSION

Symptoms of pomegranate wilt including yellowing and/or wilting of the leaves on a single branch usually in the upper crown and senescence. Brown discoloration was observed in the root, stem and branches of severely affected plants. The cross-sectioned root and stem portions of the wilted plants showed only the presence of brown discolorations in the vascular regions. The wilt incidence in Karnataka ranging from 1.14 to 62.06 Per cent.

The PCR amplification by using ITS rDNA sequence analysis and homology search through BLAST programme, it revealed that *C. fimbriata* is considered as the causal organism for wilt of pomegranate. Phylogenetic analyses placed the pomegranate isolates among members of the LAC of the *C. fimbriata* complex; specifically, to populations that appear to be native to eastern and northern South America. The colony growth of the pathogen was significantly superior on PDA followed by potato carrot agar medium. The 30°C temperature with pH of 7.00 were found to be the ideal for the growth of the pathogen. *In vitro* studies of fungicides indicated that propiconazole, propineb and mancozeb were effective against wilt pathogen. The fungal bio-agent, *T. viride* (Tv-3) and Bacillus subtilis were found to be most effective biocontrol agents against wilt pathogen. Among the nine plant extracts Pongamia leaf extract was found effective.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during the writing or editing of manuscripts.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Prasad RN, Bankar GJ, Vashishtha BB. Problems and prospects of pomegranate cultivation in arid regions. Sym. On recent advances on management of arid ecosystem held at CAZRI, Jodhpur. 1996;3:7-13.
2. Indiastat;2020. Available:https://www.indiastat.com)
3. Archana BC. Studies on leaf spot and fruit rot of pomegranate caused by Alternaria alternata (Fr.) Keissler. M.Sc. (Agri.) Thesis, Univ. Agric. Sci., Dharwad. 2012; 47-59.
4. Somasekhara YM. New record of Ceratocystis fimbriata causing wilt of pomegranate in India. Plant dise. 1999;83 (4):400-400.
5. Huang Q, Zhu YY, Chen HR, Wang YY, Liu JW and Ruan XY. First report of pomegranate wilt caused by *Ceratocystis fimbriata* in Yunnan, China. Pl. Dis. 2003; 87:1150-51.
6. Fateh FS, Kazmi MR, Ahmed I, Ashraf M, *Ceratocystis fimbriata* isolated from vascular bundles of declining mango trees in Sindh, Pakistan. Pak. J. Bot. 2006;38: 1257-59.
7. Engelbrecht CJ, Harrington TC, Alfenas A. Ceratocystis wilt of cacao a disease of increasing importance. Phytopathol. 2007;97(12):1648 -1649.
8. Somasekhara YM, Wali, SY, Bagali AN. Ceratocystis fimbriata A threatening pathogen of pomegranate *(Punica granatum* Linn.) in Northern Karnataka. Res. on Crops. 2000;1:63-66.
9. Khosla K, Gupta AK, Bhardwaj SS. Occurrence of pomegranate wilt caused by *Ceratocystis fimbriata* in Himachal Pradesh. J. Mycol. Pl. Pathol. 2011;41:1 17-19.
10. Pavithra, S Benagi, VI. *In vitro* evaluation of fungicides, botanicals and bio-agents against *Colletotrichum gloeosporioides* (Penz.) Penz. and Sacc. the causal agent of anthracnose of pomegranate. Envi. Ecol. 2012;35(2): 671-675.
11. Vincent JM., Distribution of fungal hyphae in the presence of certain inhibitors. Nature. 1947;159: 850.
12. Qian Li, Thomas C, Harrington, Douglas Mcnew, Jianqiang Li, Qiong Huang. Genetic Bottlenecks for Two Populations of Ceratocystis fimbriata on Sweet Potato and Pomegranate in China. Plant Dis. 2016;100(11):2266-2274.
13. Harrington MA, Ferreira, Somasekhara YM, Jenna V, Chase GM. An expanded concept of Ceratocystis manginecans and five new species in the Latin American Clade of Ceratocystis. Mycologia, 2023; 115(6):1-29.
14. Brito NMD, Duarte, HDSS, Buhner CDB, Auer CG and Santos AFD. Morphophysiological characterization of *Ceratocystis fimbriata* isolates from yerba mate. Ciencia Rural. 2021;51.
15. Gururaj S, Amaresh YS, Yenjerappa ST, Amaregouda A, Shreenivas AG. Cultural characteristics of *Ceratocystis fimbriata* Ell.and Halst. on different solid media causing wilt in pomegranate. Plant Archives. 2017;17(1) :51-54.
16. Rehaman A, Abbas T, Khan NA, Mehboob S. Investigations on mango sudden death syndrome affected plant parts in district muzaffargarh. Pakistan. J. Phytopathol. 2011;23(2):125-130.
17. Sharma R, Khosla K. *In vitro* evaluation of plant extracts and fungicides on Ceratocystis fimbriata (ELLIS & HALST), Incitant of pomegranate wilt. 2020;15(2): 177-181.
18. Kadam VA, Dhutraj DN, Pawar DV, Patil DD. Bio efficacy of bio agents and botanicals against Alternaria alternata (Fr.) Keissler causing leaf spot of pomegranate. Int. J. Curr. Microbiol. App. Sci. 2018;7(11):1146-1155.
19. Somasekhara YM. Evaluation of fungicides and bioagents in the management of wilt disease of pomegranate (Ceratocystis fimbriata Ell & Halst.). 2018;7(4):47-53.
20. Khan IHS, Ravindra H, Ekbote S, Narayaswamy H, Narayanaswamy P and Pradeep S. Bio efficacy of fungicides and bio agents against *Ceratocystis fimbriata* Ell. and halst. causing wilt disease of pomegranate. Int. J. Curr. Microbiol. App. Sci. 2017 6(6):2902-2907.
21. Apet KT, Sayyad AS, Wagh SS, Chavan PG. Bioefficacy of fungicides, bioagents

and phytoextracts against *Ceratocystis paradoxa,* causing pineapple disease of sugarcane. Res. J. Agric. Sci. 2015;6(6): 1266-1270.

22. Moller WJ, Devay JE. Carrot as a species selective isolation medium for *Ceratocystis fimbriata*. Phytopathol. 1968; 58: 123.

Peer-review history:
The peer review history for this paper can be accessed here:
https://www.sdiarticle5.com/review-history/116995