The Pharma Innovation Journal 2022; 11(4): 144-148

www.ThePharmaJournal.com

The Pharma Innovation

ISSN (E): 2277-7695
ISSN (P): 2349-8242
NAAS Rating: 5.23
TPI 2022; 11(4): 144-148

www.thepharmajournal.com
Received: 17-01-2022
Accepted: 31-03-2022

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Morphological characterisation of *Ceratocystis fimbriata* ell. And Halst. Causing pomegranate wilt

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Abstract
Pomegranate (*Punica granatum* L.) is one of the commercially important fruit crops of India. It is native of Iran, but spread to Mediterranean countries at an early date. Wilt caused by *Ceratocystis fimbriata* Ell. and Halst. is currently one of the most important diseases affecting pomegranate. To study the morphological variability of fifteen pathogenic isolates, characteristics such as, colony color, type of colony growth and type of margin and growth rate were assessed on potato dextrose agar. Pathogen produced septate mycelium which was initially whitish grey and changed to dark grey on potato dextrose agar. With respect to colony growth, all the isolates exhibited flat growth and none of them were fluffy. When isolates observed for colony margin, all isolates exhibited uniform colony margin except isolate Cf-7 and most of the isolates had taken 15-24 days to cover the entire Petri plate. The pathogen was observed for the production of different spores such as endoconidia, aleurioconidia, perithecia and ascospores. Endoconidia were hyaline, cylindrical and measured 9.10-25.80 × 3.50-7.50 µm and aleurioconidia were thick walled pyriform with size of 8.70-17.00 × 8.20-12.00 µm. Black colored perithecia with globose base bearing long neck was observed with size of 480.00-880.00 × 20.00-38.00 µm exuding small, hyaline and hat shaped ascospores from the apex of the perithecium which measures 3.60-5.00 × 2.50-4.00 µm.

Keywords: Pomegranate wilt, Ceratocystis fimbriata, morphological, characterization

Introduction
Pomegranate (*Punica granatum* L.) is one of the important ancient fruit crops belonging to the botanical family punicaceae. It is a commercial fruit crop of both tropical and subtropical countries. The fruit is symbolic for its cool, refreshing juice and known to possess pharmaceutical and therapeutic properties. Pomegranate production has attracted several farmers due to its wider adaptability, drought tolerant capacity, hardy nature, relatively low cost of cultivation, higher yields, excellent keeping quality and export potential (Jamadar *et al*., 2009). India is one of the major producers of pomegranate in the world. It is commercially cultivated in the states of Maharashtra, Karnataka, Gujarat, Andhra Pradesh, Madhya Pradesh, Rajasthan and Tamil Nadu. Karnataka is the second leading producer and the crop has extended across different districts such as, Chitradurga, Ballari, Tumakuru, Vijayapura, Bagalkote, Koppala, Belagavi, Davanagere, Bengaluru and Kalaburagi (Anon., 2018) [2]. In recent years, the successful cultivation has met with different traumas mainly pest and diseases. Among the diseases infecting pomegranate, wilt caused by *Ceratocystis fimbriata* Ell. and Halst. is an important disease which results in complete wilting of the plant. At present, the crop is severely affected by wilt pathogen and wilting severity is increasing day by day at faster rate. The disease is characterized by yellowing of foliage of one or few branches of a plant. Later progression of disease resulted in complete yellowing of entire plant and finally leads to wilting. The partial wilting of the plant with drying and death of some branches were the common symptoms. In severe cases, the defoliation and complete wilting of plants were observed within 2-3 months. Vertical section of affected plant parts near the root region reveals dark grayish brown discoloration which is the typical symptom of wilt disease. Wilt pathogen probably survives as mycelia within host plant or thick-walled aleurioconidia in the soil or in plant debris. The fungus survives in infected pomegranate plant up to 190 days and in soil for at least four months (Somasekhara *et al*., 2009) [8]. Secondary spread of the pathogen generally takes place through infected seedlings, irrigation and rain water, root contact, insects, implements, pruning and budding tools.

Variability in pathogen was studied using morphological characteristics to focus on the existence of variation in *C. fimbriata* isolates collected from different locations during the survey which serves as key for management aspects.

Material and Methods

Field survey

Roving field survey was undertaken in the major pomegranate growing southern districts of Karnataka during the year 2018-19 and 2019-20 and collected the different isolates of *Ceratocystia fimbriata* (Table 1).

Isolation of the pathogen

Pathogen isolation was done by using carrot bait technique (Moller and Devay, 1968) [5] where tissue bits were enclosed in a cavity hollowed out of the inner face of a pair of disks cut from carrots. The disks were fastened together with a sterile rubber band and incubated at high Relative Humidity (RH) for 4 days at 26±1 °C. The presence of fungus was apparent after 4-5 days during which typical perithecia and ascospores were formed. Later, a portion of the fungus was transferred into Petri plate containing solidified PDA to allow the full development of the fungus.

Tissue isolation method

The samples with typical wilt symptoms brought from survey were cut into small bits of size 0.5 to 1.0 cm. The tissue bits were initially surface sterilized with 1% sodium hypochlorite (NaOCl) for 1 min and washed with sterile distilled water successively for 3 times (30 seconds each time). Later, the bits were placed on sterile tissue paper to remove excess moisture and finally placed on solidified potato dextrose agar medium in sterile Petri plate and incubated at 26±1 °C temperature until the growth initiates. Culture of *C. fimbriata* was purified by following standard hyphal tip method and then purified cultures were maintained on potato dextrose agar slants and kept in refrigerator at 4 °C for further use.

Table 1: Isolates of *Ceratocystis fimbriata* collected from different southern districts of Karnataka

Sl. No.	Isolate	Name of the place		
		Village	Taluk	District
1	Cf-1	Pura	Devanahalli	Bengaluru Rural
2	Cf-2	Harohalli		
3	Cf-3	Vijayapura		
4	Cf-4	Yaluvahalli		
5	Cf-5	Kalenahalli	Bengaluru North	Bengaluru Urban
6	Cf-6	GKVK, Hebbal		
7	Cf-7	Uyyalappanahalli	Kanakapura	Ramanagara
8	Cf-8	Jakkalli	Kollegala	Chamarajanagara
9	Cf-9	Rangapura	Sira	Tumakuru
10	Cf-10	Thogaragunte		
11	Cf-11	Dharmapura	Hiriyur	Chitradurga
12	Cf-12	Javagondanahalli		
13	Cf-13	Maskal		
14	Cf-14	Ramajjanahalli	Hosadurga	
15	Cf-15	Kurubarahalli		

Morphological characterization of the pathogen

Initially, pathogen was identified based on morphological characteristics in culture plate and through microscopic observation. Different Spores *viz*., endoconidia, aleurioconidia, perithecia and ascospores were observed under microscope at 10x, 40x and 100x objectives. In addition, to confirm the identity of the pathogen, molecular characterization was also carried out by ITS (Internal Transcribed Spacer region) sequencing using the ITS region primers set ITS-1-F and ITS-4-R.

Morphological characters were studied from 15 days old culture of *C. fimbriata*. Mycelial disc of 5 mm diameter was cut from periphery of actively growing culture and transferred aseptically to a 90 mm Petri dish containing 20 ml of potato dextrose agar and incubated at 26±1 °C for 15 days. The pathogen was observed for the production of different spores such as endoconidia, aleurioconidia, perithecia and ascospores. A small quantity of culture was taken on a clean glass slide using sterilized needle and measured length and breadth of each spore in µm with the help of fluorescent microscope. Spores 'picked up randomly to determine the diameter and three observations were taken for each spore type. The pathogen was also characterized for its colony color, type of margin, growth pattern and growth rate on potato dextrose agar.

Results

Morphological characterization of the pathogen

To study the morphological variability of fifteen isolates, characteristics such as colony color, type of colony growth and type of margin and growth rate were assessed on potato dextrose agar and results are presented in Table 2 and 3.

Isolates were found diverse with respect to their colony color as they existed in different colors such as whitish grey, greenish grey, light grey, greyish, grey and dark grey. Among fifteen isolates, six isolates (Cf-3, Cf-5, Cf-8, Cf-11, Cf-12 and Cf-15) were grey in color, three were dark grey (Cf-6, Cf-10 and Cf-13), two were light grey (Cf-2 and Cf-9), isolates Cf-4 and Cf-7 were greyish, isolate Cf-1 was whitish grey and isolate Cf-14 was greenish grey. With respect to colony growth, all the isolates exhibited flat growth and none of them were fluffy. When isolates observed for colony margin, all isolates exhibited uniform colony margin except isolate Cf-7 which was irregular. Isolates were also assessed for their growth rate and found that, isolates had taken 15-35 days to cover entire Petri plate and minimum days (15 days) had taken by isolate Cf-5 and was found on par with isolates, Cf-15 (17 days), Cf-8 (18 days) and Cf-2 (19 days) whereas, maximum days had taken by isolate, Cf-14 (35 days) followed by isolates Cf-1 and Cf-13 which had taken 30 days to cover entire Petri plate.

Pathogen produced septate mycelium and was initially whitish grey and changed to dark grey on potato dextrose agar. The endoconidia and aleurioconidia were produced after 3-5 days of incubation and perithecium produced after 10-15 days. Endoconidia were hyaline, cylindrical and formed endogenously in hyphae. Aleurioconidia were thick-walled resting spores, pyriform or globose in shape and brown in color usually borne singly or in chain. The black colored perithecia with a globose base bearing long neck were observed which exude small, hyaline and hat shaped ascospores from the apex of the perithecium neck which are the sexual spores of the fungus (Plate 1a & 1b).

Diversity in morphological characteristics such as length and breadth of endoconidia, aleurioconidia, perithecia and ascospores were measured using fluorescent microscope (Table 3). Results found that, all fifteen isolates showed variability with respect to spore size.

The length of endoconidia was ranged from 9.10 to 25.80 µm

and breadth was ranged from 3.50-7.50 µm. Isolate Cf-13 was exhibited maximum size of endoconidia (25.80 µm × 7.50 µm) followed by isolate, Cf-8 (24.20 × 7.30 µm) and minimum sized endoconidia were observed in isolate, Cf-15 (9.10 µm × 3.80 µm).

The length of aleurioconidia was ranged from 8.70-17.00 µm and breadth was ranged from 8.20-12.00 µm. Isolate Cf-10 was recorded maximum size of aleurioconidia (17.00 × 12.00 µm) followed by isolate Cf-11 (16.20 × 10.20 µm) and minimum sized aleurioconidia were produced in isolate Cf-5 (8.70 × 8.20 µm).

The length of perithecia was ranged from 480.00-880.00 µm and breadth was from 20.00-38.00 µm. Isolate Cf-2 was recorded maximum size of perithecium (880.00 µm × 38.00 µm) followed by isolate, Cf-5 (668.00 × 36.30 µm) and minimum sized perithecia were found in isolate Cf-11 (480.00 µm × 20.00 µm).

The length of ascospores was ranged from 3.60-5.00 µm and breadth was ranged from 2.50-4.00 µm. Isolate Cf-3 was recorded maximum sized ascospores (5.00 × 4.00 µm) followed by isolate, Cf-4 (4.80 × 3.90 µm) and minimum sized ascospores were found in isolate Cf-8 (3.60 × 2.50 µm).

Table 2: Cultural characteristics of different isolates of *Ceratocystis fimbriata* on potato dextrose agar

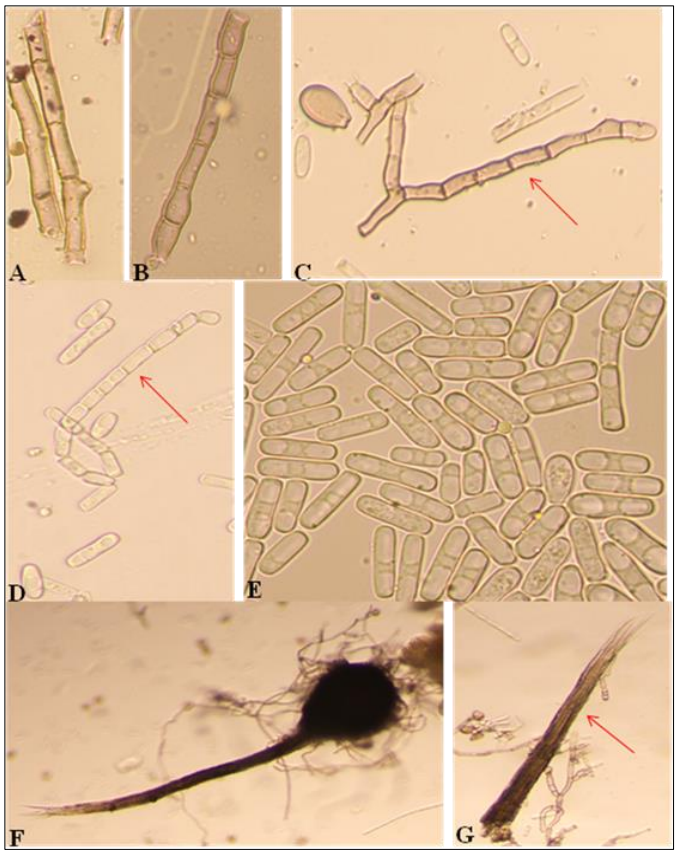
Sl. No.	Isolate	Colony colour	Type of colony growth	Type of margin	Growth rate				Days taken for complete growth
					Growth at 10 DAI	Growth at 15 DAI	Growth at 20 DAI	Growth at 25 DAI	
1	Cf-1	Whitish grey	Flat	Uniform	3.63	6.13	7.13	7.83	30
2	Cf-2	Light grey	Flat	Uniform	5.76	8.60	9.00	9.00	19
3	Cf-3	Grey	Flat	Uniform	4.06	6.67	9.00	9.00	20
4	Cf-4	Greyish	Flat	Uniform	5.23	8.30	8.73	9.00	23
5	Cf-5	Grey	Flat	Uniform	6.80	9.00	9.00	9.00	15
6	Cf-6	Dark grey	Flat	Uniform	4.33	7.26	8.32	9.00	24
7	Cf-7	Greyish	Flat	Irregular	4.53	7.36	7.98	9.00	26
8	Cf-8	Grey	Flat	Uniform	4.93	8.23	9.00	9.00	18
9	Cf-9	Light grey	Flat	Uniform	6.20	8.83	8.92	9.00	23
10	Cf-10	Dark grey	Flat	Uniform	4.20	7.50	9.00	9.00	19
11	Cf-11	Grey	Flat	Uniform	5.20	7.00	8.60	9.00	22
12	Cf-12	Grey	Flat	Uniform	4.86	7.13	8.86	9.00	21
13	Cf-13	Dark grey	Flat	Uniform	2.63	3.83	5.33	6.46	30
14	Cf-14	Greenish grey	Flat	Uniform	4.30	5.26	5.91	6.56	35
15	Cf-15	Grey	Flat	Uniform	5.96	8.80	9.00	9.00	17
								S.Em±	1.64
								CD @ 1%	4.81

DAI: Days after inoculation

Table 3: Morphological characteristics of different isolates of *Ceratocystis fimbriata* on potato dextrose agar

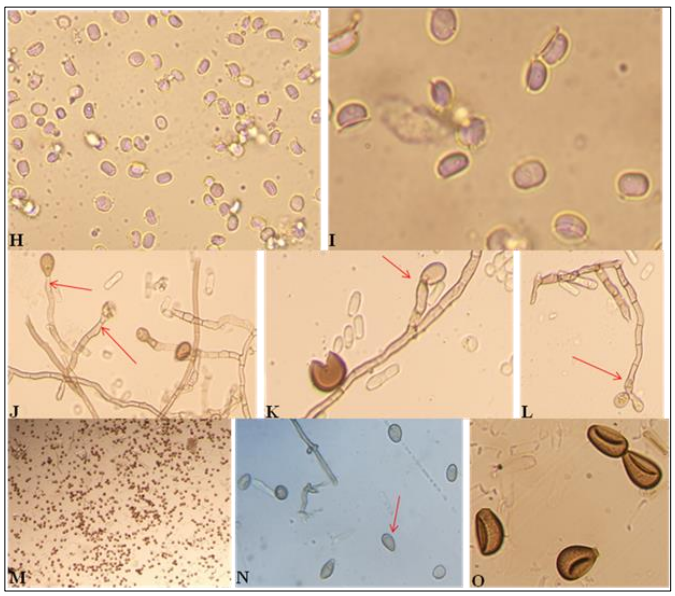
Sl. No.	Isolate	Endoconidia (L×B) (µm)	Aleurioconidia (L×B) (µm)	Perithecia (L×B) (µm)	Ascospores (L×B)* (µm)
1	Cf-1	13.50×4.50	10.80×9.70	560.18×32.14	4.10×3.20
2	Cf-2	21.50×5.20	15.70×9.30	880.00×38.00	4.40×3.10
3	Cf-3	12.90×4.70	11.30×8.50	538.30×26.40	5.00×4.00
4	Cf-4	22.80×7.10	13.20×8.80	543.80×32.50	4.80×3.90
5	Cf-5	9.40×3.50	8.70×8.20	668.00×36.30	4.30×2.90
6	Cf-6	11.20×6.40	10.60×9.80	645.90×29.70	4.50×3.80
7	Cf-7	15.10×5.80	15.50×8.40	528.50×29.12	4.10×3.00
8	Cf-8	24.20×7.30	12.10×9.10	589.70×34.70	3.60×2.50
9	Cf-9	18.20×6.70	14.60×9.60	532.80×29.50	4.70×2.80
10	Cf-10	20.30×5.90	17.00×12.00	501.20 ×23.40	3.80×2.60
11	Cf-11	23.10×6.20	16.20×10.20	480.00×20.00	3.70×2.70
12	Cf-12	14.70×4.10	12.80×8.90	548.00 ×28.80	4.00×3.60
13	Cf-13	25.80×7.50	11.90×9.00	530.50×30.80	4.60×3.30
14	Cf-14	17.80×6.10	14.10×9.50	541.40×31.90	3.90×3.40
15	Cf-15	9.10×3.80	13.90×8.30	523.30×24.10	4.20×3.50

* L × B = (Length × Breadth)



A, B & C = Fungal hyphae (100x); D = Chain of endoconidia (40x); E = Endoconidia (100x); F = Perithecium (10x); G = Perithecium neck/ostiole (10x)

Plate 1a: Spore morphology of *Ceratocystis fimbriata* infecting pomegranate



H & I = Hat shaped ascospores (100x); J, K & L = Conidiophore bearing aleurioconidia (40x); M = Aleurioconidia (10x); N = Aleurioconidia (40x); O = Aleurioconidia (100x)

Plate 1b: Spore morphology of *Ceratocystis fimbriata* infecting pomegranate

Discussion

Morphological variability of different isolates of *Ceratocystis fimbriata*

In the present investigation, morphological characteristics were varied among the *C. fimbriata* isolates. The variation was observed with respect to colony color (whitish grey, greenish grey, light grey, greyish, grey and dark grey), type of margin (uniform to irregular) and growth rate (slow to fast growing). Based on colony color, isolates were categorized into six groups *viz*., whitish grey (1 isolate), greenish grey (1 isolate), light grey (2 isolates), greyish (2 isolates), grey (6 isolates) and dark grey (3 isolates). Based on type of margin, isolates were categorized into two groups *i.e.,* irregular and uniform/regular. Fourteen isolates showed regular type of margin whereas one isolate was irregular. The rate of growth was closely observed in the isolates and found that, isolates had taken 15-35 days to cover entire Petri plate. Minimum days (15 days) were taken by isolate Cf-5 which was found on par with isolates Cf-15 (17 days), Cf-8 (18 days) and Cf-2 (19 days) and the isolates were considered as fast growing. Isolate Cf-14 had taken maximum days (35 days) followed by Cf-1 and Cf-13 each had taken 30 days and were grouped as slow growing and remaining isolates had taken 20 to 26 days and were considered as moderately growing isolates. Results were found similar with Raja (2017) [7] who worked on *Ceratocystis fimbriata* causing pomegranate wilt. They categorized isolates collected from different locations of Karnataka into three groups based on colony color *i.e*., greyish, light grey and brown. Thirty two isolates grouped as greyish, ten isolates as light grey and eight isolates as brown color. Similar results with respect to variation in type of margin were also reported by Raja (2017) [7] in which out of fifty isolates, forty two were having regular type of margin and eight isolates exhibited irregular margin. The findings are also in accordance with Hussain *et al*. (2012) [3] who classified the isolates of *Sclerotium rolfsii* based on morphological variation as fast growing, moderately and slow growing isolates and found that, isolates AT-1, AT-2 and RW-2 as fast growing, isolates, SR-1, CH-1 and DL-2 as intermediate and SR-2, CH-2, CH-3, DL-1, AT-3 and RW-1 were grouped under slow growing isolates.

In the present study, all the fifteen isolates showed variability with respect to size of endoconidia, aleurioconidia, perithecia and ascospores. The present findings are in accordance with earlier workers. Xu *et al*. (2011) [9] conducted a study on *C. fimbriata* and reported that, endoconidia measured 9.20 to 29.60 × 3.10 to 6.80 µm, aleurioconidia were brownish, thick walled, near globose and measured 8.70 to 18.10 × 8.20 to 10.70 µm, perithecia were dark brown to black, globose, measured 90.80 to 149.80 µm in diameter and had a long thin neck of 254.40 to 533.80 µm long through which ascospores exuded. Ascospores were small, hyaline; hat shaped measured 3.70 to 6.50 × 3.10 to 5.70 µm and accumulated in a sticky matrix at the tip of ascomal neck. Similarly, Alam *et al*. (2016) [1] reported that, perithecia were dark brown to black and the base was 153 to 281 µm in diameter. Ascomatal necks were 514 to 653 µm long, dark brown to black, lighter in color at apices, tapering from base (25 to 48 µm diameter) to apex (14 to 26 µm diameter). Piveta *et al*. (2016) [6] reported that, perithecia bases were dark, globose measured 90-260 µm wide with straight necks which measured 100-825 µm long. Ascospores measured 4-6 × 3-5 µm and aleurioconidia measured 8-17 × 6-12 µm.

Conclusion

Among fifteen isolates, six isolates (Cf-3, Cf-5, Cf-8, Cf-11, Cf-12 and Cf-15) were grey in color, three were dark grey

(Cf-6, Cf-10 and Cf-13), two were light grey (Cf-2 and Cf-9), isolates Cf-4 and Cf-7 found greyish color, isolate Cf-1 was whitish grey and isolate Cf-14 was greenish grey. With respect to colony growth, all the isolates exhibited flat growth. When isolates observed for their colony margin, all exhibited uniform colony margin except isolate Cf-7. Isolates were also assessed for growth rate in which, isolate Cf-5 was fast growing (15 days) and was on par with isolates, Cf-15 (17 days), Cf-8 (18 days) and Cf-2 (19 days). In general, most of the isolates were found grey in color with flat type of colony growth and uniform margin and took 15-24 days to cover the entire Petri plate.

The endoconidia were hyaline, cylindrical formed endogenously in hyphae which measured 9.10-25.80 × 3.50-7.50 µm and isolate Cf-13 exhibited maximum size. Aleurioconidia were thick-walled resting spores, Pyriform in shape, brown in color borne singly or in chain measured 8.70-17.00 × 8.20-12.00 µm and maximum sized spores were produced by isolate Cf-10. The black colored Perithecia with a globose base bearing long neck were observed with size of 480.00-880.00 × 20.00-38.00 µm and isolate Cf-2 produced maximum sized spores and perithecium exudes small, hyaline and hat shaped ascospores from the apex which measures 3.60-5.00 × 2.50-4.00 µm and isolate Cf-3 produced maximum sized spores.

Acknowledgement

I am deeply indebted to DST-INSPIRE, Ministry of Science and Technology, Government of India for financial assistance to carry out this work.

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