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Assessment of Mycelial Growth of Pathogenic Variable FOC Isolates on Different Nutritional and Physiological Conditions

Richa*[1], Rishi Saxena[1], Manoj Srivastava[2] and H. V. Singh[3]

[1] Bundelkhand University, Jhansi - 284 128, Uttar Pradesh, India
[2] ICAR – Indian Grassland and Fodder Research Institute, Jhansi - 284 003, Uttar Pradesh, India
[3] ICAR- Nationally Bureau of Agriculturally Important Microorganism, Kushmaur - 275 103, Uttar Pradesh, India

Abstract

Chickpea is a major pulse crop, largely cultivated for human and animal consumption in the world. In India: many states are facing low production, due to wilt disease which is a major biotic stress caused by wilt pathogen. In this study three isolates of wilt pathogen, isolated from Datia district of Bundelkhand region were studied for their pathogenic variability (in pot experiment) and mycelial growth of wilt pathogen in different physiological (*In vitro*) condition. Present findings indicated that all the three representative isolates of *Fusarium oxysporum* f. sp. *ciceri* showed different mycelial growth, when expose to different nutritional and physiological conditions. Maximum FOC mycelial growth and sporulation for all the isolates observed after 7 DAI at 25 °C and 30 °C and reduced significantly below 15 °C and above 35 °C. Different culture media significantly influence mycelial growth and sporulation of FOC isolates. FOC isolates nurtured well on CZA and PDA medium. Among six culture media maximum number of macro conidia and microconidia observed on CZA and PDA media. FOC growth was found most appropriate in pH 6 and pH 7. In addition, MPFOC13 and MPFOC14 FOC isolates found strong pathogenic on response on chickpea variety JG-62 whereas MPFOC11 was found to be moderate pathogenic.

***Key words*:** Chickpea, *Fusarium oxysporum* f. sp. *ciceri*, Nutritional media, Pathogenicity, pH, Temperature

Chickpea (*Cicer arietinum* L.), is a cool-season, protein rich pulse crop. Which ranks first in the Mediterranean region, South Asia and the third-most major grain legume crop worldwide. India shares for over 75 percent of total world's production of chickpeas [1]. In India, 10.57 million ha of chickpeas are cultivated, yielding 11.16 million tonnes at a productivity of 1056 kg per hectare [2]. Among various biotic and abiotic variables that contribute to the low output of chickpeas, still the main cause of low output is the chickpea plant's susceptibility to a variety of fungi from seedling to maturity. According to reports, more than 52 diseases are known to damage chickpea [3]. One of them, wilt disease is a widespread soil-borne disease caused by *Fusarium oxysporum* f. sp. *ciceri* that has been recorded from numerous locations in India, with an intensity ranging from 10 to 100 percent [4]. Also there have been reports of the chickpea wilt disease being widely dispersed in close to 32 different countries around the world [5-6].

With more than 120 reported formae speciales capable of inflicting vascular wilt illnesses on numerous agricultural crops, *F. oxysporum* exhibits a high degree of host specificity. The aptitude to parasitize plant roots, typically without causing any symptoms, is a common feature of *F. oxysporum* strains. On the basis of virulence to a specific group of diverse host cultivars that differ in disease resistance, formae speciales are frequently further subdivided into races [7]. Previous reports revealed that it is essential to have knowledge about the assortment, appearance and inheritance of the resistance as well as the isolate [8]. According to Haware and Nene [9], the significant pathogenic heterogeneity in the FOC may hinder the efficacy of resistance. Moreover, the poor agronomic traits of resistant cultivars have impeded their development [10].

Generally filamentous fungi produce mycelium during somatic growth to give nutrients so they can stay live, however conidia are specific structures that are often responsible for spreading and environmental perseverance [11] and serve as the asexual reproduction entities for a variety of fungal plant diseases and are crucial for both fungus growth and host infection [12]. *Fusarium oxysporum* is identify as an anamorphic species by physical characteristics commonly shared by both pathogenic and non-pathogenic strains of [13]. The three different forms of asexual spore (Microconidia, macroconidia, and Chlamydospores) are produced by *Fusarium oxysporum*. Formae speciales of *F. oxysporum* strains have been created based on their pathogenicity toward a certain host or group of hosts [14].

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Correspondence to: Richa, Bundelkhand University, Jhansi - 284 128, Uttar Pradesh, India, Tel: +91 9450137241; E-mail: richasoni10@gmail.com

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In view of the above facts, the present research work was aimed to carry out comprehensive investigation by study of mycelial growth and sporulation of *Fusarium oxysporum* f. sp. *ciceri* isolates in different nutritional sources as well as in different physiological conditions and confirmation of pathogenicity-by-pathogenicity test by greenhouse bio essay.

MATERIALS AND METHODS

Diseased chickpea plants (infected with fusarium wilt disease), were collected for the isolation of wilt pathogens from Rajpura village of Datia district, Bundelkhand region. Plants which began to wilt and their leaves became light and more greyish green in shade were taken from the farmer's fields [15]. Examination of the roots revealed that inner vascular tissue which is made up of pith and xylem, was brown to black in colour in both seedling and adult plant infections. Such withered samples were gathered and taken to a lab for isolation. Three FOC isolates were selected as representative isolates and further studied for their morphological characteristics, pathogenic variability (in pot experiment), mycelial growth on different nutritional, physiological conditions (*In vitro*).

Isolation and purification of wilt pathogen

For the isolation of wilt pathogen from diseased chickpea plant, initially roots of diseased plant were washed with tap water to remove dust particle from root surface [16]. After that diseased root was cut into small section, and subjected to surface sterilization by putting these small parts (for one minute) in with 1% per cent mercuric chloride (NaOCL) solution, after that finally washed with distilled water (2 to 3 minute, repeated for three times). Further the excess water was removed from these root pieces by the help of sterilized blotter papers. These root sections transferred on Petri plates containing PDA medium, after that these petri plates were incubated at 27± °C for the growth of the pathogen. FOC culture were further observed in stereoscopic (Model: Leica), and compound microscope (Model: Leica) for the identification of the desired pathogens. Further this pathogen culture was purified by single spore culture technique. For further study culture of these purified isolates were preserved in PDA slants at 4 °C [8]. Isolated FOC colonies were identified with the help of relevant literature [17].

In present study three representative FOC isolates isolated from Datia district of Bundelkhand region, were observed on PDA media for their morphological variability. FOC isolates were categorized on the basis of colony pigmentation and studied for colony shape, texture, margin and conidia (occurrence, type, size and shape).

Effect of different nutritional medium on mycelial growth of Fusarium oxysporum f. sp. ciceri

In present study six different nutritional medium used as culture media viz. Potato dextrose agar (PDA), Czapek's dox agar (CDA), Modified Czapek's dox agar (MCA), Corn meal agar (CMA), Oat meal agar (OMA), RB (Rose Bengal Agar) to find out their effect on mycelial growth and sporulation of FOC isolates.

All the six media were sterilized in autoclave at 15 lbs/inch^2 pressure for 20 min, and cooled at room temperature. Cooled media were poured in sterilized Petri plates and allowed to solidify at room temperature. After solidification of the poured petri plates, a five mm mycelial disc from actively growing pure culture of FOC isolate was inoculated by placing in the centre (each replicated thrice). These plates were incubated at 27 ± 2^{0}C. Effect of each culture media on mycelial growth were observed at a seventh day and that of sporulation at fourteen days of incubation. Conidial production was determined on the basis of microscopic observations [18].

Physiological variability assessment of FOC isolates

Effect of temperature on mycelial growth and sporulation of FOC isolates

All three representative FOC isolates were inoculated on PDA media which used as basal media and incubated at six different temperatures viz., 10, 15, 20, 25, 30 and 35 °C to determine the effect of temperature on radial growth and sporulation of *Fusarium oxysporum* f. sp. *ciceri* by application of biochemical oxygen demand (BOD) incubator. Three replications were maintained for each isolate. PDA containing plates were inoculated by placing a five mm mycelial disc of actively growing FOC culture, further these inoculated plates were incubated in BOD incubators (set at relevant temperature regimes). At seventh day after inoculation mycelial growth of FOC isolates was recorded [18].

Effect of pH levels on mycelial growth and sporulation of Fusarium oxysporum f. sp. ciceri

In recent study effect of six different pH levels (hydrogen ion concentration) on growth of three FOC isolates was studied by using PDA as basal medium. 200 ml PDA medium was poured in glass beakers (250 ml) and its pH levels (4.0, 5.0, 6.0, 7.0, 8.0 and 9.0) were independently adjusted by using pH meter (make: MAC, Delhi). The PDA medium was adjusted with different level of pH and maintained by adding 0.1 N NaOH or 0.1N HCl and autoclaved at 15 PSI for 20 min. Autoclaved and cooled PDA medium with various pH levels was poured (20 ml / plate) separately in sterile Petri plates (with three replication of each isolates). With the help of a cork borer (flame sterilized), a mycelial disc (Five mm diameter) was cut from the margin of actively growing culture of *Fusarium oxysporum* f. sp. *ciceri* and then placed into the centre of the Petri plates containing solidified different pH levels PDA medium. The petri plates were placed in a BOD at 27 °C. Mycelial radial growth of FOC isolates was observed and recorded after two days of incubation till completely cover all the Petri plates. Sporulation of FOC isolates on different pH levels was observed after seven days of incubation.

Pathogenic variability assessment

Pathogenic variability assessment of three FOC isolates were examined on wilt prone chickpea cultivar JG-62 in pot house, by using soil inoculation techniques [19]. The pots were filled with 500 gm sterilized soil. In order to get a mass inoculum of FOC isolates, sorghum seed were soaked (overnight) in water and autoclaved. Autoclaved sorghum seed further inoculated with representative FOC isolates (two mycelial bit of actively growing culture) incubated for 10 days, prepared inocula were incorporated into the sterilized soil in Pot. Five to six seeds of chickpea cultivar JG-62 were sown in one pot (each pot have three replications). Prior to sowing seeds were surface sterilized with 1% per cent mercuric chloride (NaOCL) solution for 30 seconds and rinsed by sterile distilled water for three times. Observations for wilt disease were observed and recorded at 30, 45 and 60 DAI (days after inoculation). However, virulence of the tested FOC isolates were notated by considering wilt incidence only at 60 DAI. Pathogenicity of each FOC isolate was established and Koch's postulates verified [20].

Statistical analysis

The research experiment was laid out in Completely Randomized Design (CRD) and three independent observations of each sample for each test were taken and mean of this observation was used for the analysis. Data were analyzed using online OPSTAT software, a Statistical Software Package for agricultural research workers [21].

RESULTS AND DISCUSSION

In present study three FOC isolates from Datia district (Rajpura village) of Bundelkhand region were isolated and studied for their pathogenic variability (in Pot house experiment) and their mycelial growth in different nutritional and physiological condition (in vitro). Present findings shows that all isolates exhibited morphological variation i.e., colony characteristics such as colour, shape, margin and texture. In present study FOC culture shows different pigmentation which were white Floccose, white violet felted and white with violet floccose. It was observed that colony shapes were irregular to regular whereas colony margins of FOC isolates were found irregular, entire and wavy with fluffy, flat/velvet colony textures (Table 1).

Table 1 Morphological characteristics of FOC isolates

FOC code	District	Village	Shape	Mycelium colour	Margin	Texture	Spores			
							Microconidia	Macroconidia	Chlamydospores	Sporulation
MPFOC11	Datia	Rajpura	Irregular	White floccose	Lobate	Fluffy	Present	Present	Present	+++
MPFOC13	Datia	Rajpura	irregular	White violet felted	Lobate	Velvety	Present	Present	Present	++++
MPFOC14	Datia	Rajpura	Circular	White with violet floccose	Entire	Fluffy	Present	Present	Present	+++

Variations in the size of micro and macroconidia (with three replications) was also observed. The minimum length of micro conidia ranged from 2.22 to 21.69 µm and the maximum length ranged from 12.378 to 21.69 µm, whereas the minimum width of microconidia was ranged from 1.54 to 1.81 µm and maximum width ranged from 3.22 to 5.97 and septa ranged between 0-2. Whereas the minimum and maximum length of macro conidia ranged from 20.54-23.61 and 31.56-33.59 respectively, whereas minimum and maximum width of macro conidia ranged from 1.54-3.09 µm and 2.96-3.81 µm respectively, the number of septation ranged from 2-3. The largest size of the micro-conidia was obtained from the isolate MPFOC14 with (12.37×3.08 µm) and the smallest size was from isolates MPFOC11 (2.22×1.86 µm). Whereas, the biggest size (33.59×3.09 µm) of the macro-conidia was found from the isolates MPFOC14 and the smallest size of (20.54×2.44 µm) macroconidia were obtained from isolates MPFOC13 (Table 2).

The current findings were well supported by Dubey *et al*. [22] and Chauhan [23], they found high variability while studying FOC isolates in the form of mycelium, colony pigmentation, toxin production and as well as colony growth pattern, size of colony and in their pathogenicity [22-23].

Table 2 Spore size measurement of FOC isolates

FOC code	District	Village name	Microconidia					Macroconidia					Chlamydospores
			Length		Width		Septa	Length		Width		Septa	
			Min	Max	Min	Max		Min	Max	Min	Max		
MPFOC11	Datia	Rajpura	2.225	21.657	1.549	3.81	0-2	23.618	31.56	1.549	3.81	3-4	Present
MPFOC13	Datia	Rajpura	3.225	12.378	1.76	3.225	0-2	21.657	33.596	3.095	3.795	3-4	Present
MPFOC14	Datia	Rajpura	3.34	21.69	1.811	5.97	0-2	20.54	32.56	2.11	2.96	3-4	Present

*Based on 100 observations

Pathogenic variability assessment

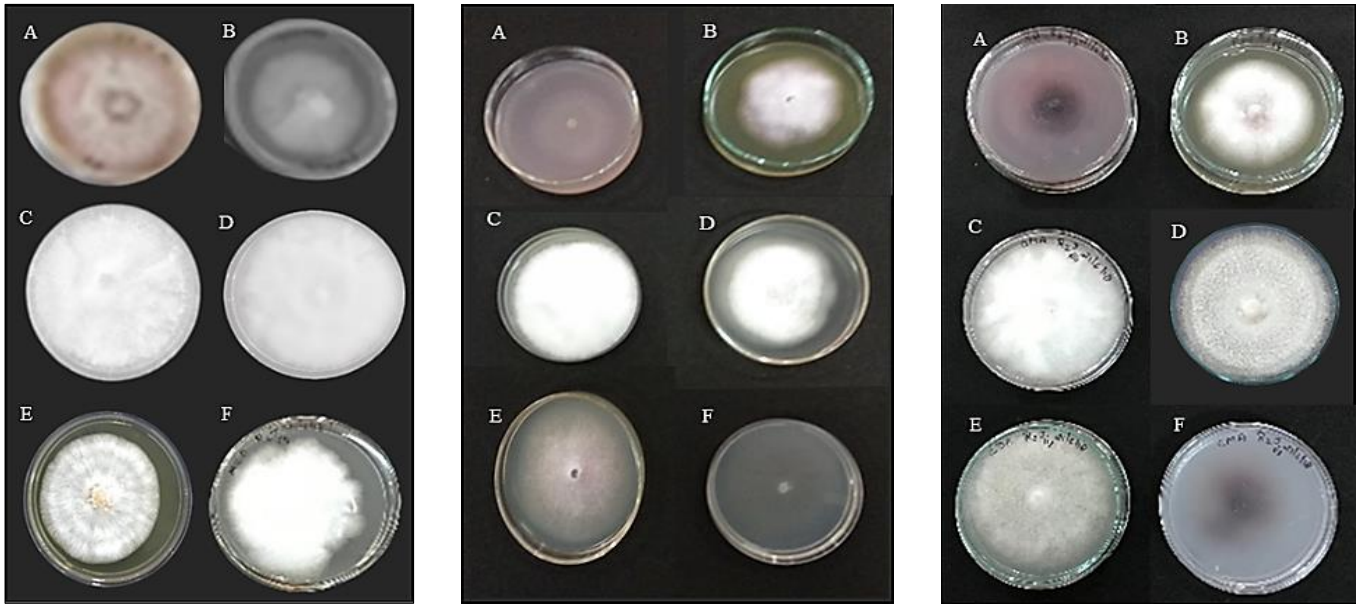
The pathogenic diversity present in representative isolates of *Fusarium oxysporum* f. sp. *ciceri* was investigated in an experiment. The highly wilt prone genotype JG-62 was used to screen three isolates of *Fusarium oxysporum* f. sp. *ciceri* in sick soil using the pot culture method. Present finding revealed that MPFOC 13 and MP FOC 14 exhibit strong pathogenic disease symptoms in JG-62 cultivar. Whereas MPFOC11 was observed with moderate pathogenic characteristics in cultivar JG-62 (Table 3).

Table 3 Performance of FOC isolates through pathogenicity test on cultivar JG-62

FOC Code	Pathogenicity	Symptoms
MPFOC11	Moderate pathogenic isolates	Yellowing of leaf
MPFOC13	Strong pathogenic isolates	Wilting with yellowing of leaf
MPFOC14	Strong pathogenic isolates	Wilting with yellowing of leaf

Culture variability assessment on different media

The findings of present work witnessed that all of the six-culture media stimulated better growth (Fig 1) and sporulation of all three *Fusarium oxysporum* f. sp. *ciceri* isolates. The mean colony diameter / mycelial growth was recorded after seventh day incubation. The maximum mean mycelial floccus growth of test pathogen was observed on Czapek's dox agar medium in MPFOC13 (81.333mm), which is followed by mycelial growth of MPFOC13 (80.167 mm) on Potato dextrose agar. Comparatively thin and minimum mean mycelial growth in MPFOC14 (53.66mm) was observed on Rose Bengal medium, Corn Meal Agar thin mycelium (54.833 mm), Rose Bengal agar (54.00 mm) medium, thrice of which were at par (Table 4, Fig 2).



MPFOC11 MPFOC13 MPFOC14

Fig 1 Mycelial growth of three FOC isolates after seventh day incubation on different media
(A) Rose Bengal (RB), (B) Oat Meal Agar (OMA), (C) Potato Dextrose Agar (PDA), (D) - Czapek's Dox Agar (CZA), (E) Modified Czapek's Dox Agar (MCA), (F) Corn Meal agar (CMA)

Table 4 Nutritional variability assessment of three FOC isolates on different media

Treatment*	MPFOC11		MPFOC13		MPFOC14		Sporulation**
	Mean	S.E.	Mean	S.E.	Mean	S.E.	
RB	61.000	0.577	54.000	0.577	53.667	0.333	+
OMA	68.000	0.577	63.667	1.202	57.167	0.441	++
PDA	77.167	0.441	80.167	0.441	71.000	0.577	+++
CZA	77.833	0.441	81.333	0.601	73.000	0.577	+++
MCA	73.333	0.726	72.000	0.577	57.000	0.577	+++
CMA	68.333	0.333	68.667	0.334	54.833	0.333	++
C.D.	1.656		2.120		1.514		
SE(m)	0.531		0.680		0.486		
SE(d)	0.752		0.962		0.687		
C.V.	1.297		1.684		1.377		

*RB-Rose Bengal, OMA- Oat Meal Agar, PDA- Potato Dextrose Agar, CZA- Czapek's Dox Agar, MCA- Modified Czapek's Dox Agar; CMA-Corn Meal agar
**+++High; ++Moderate; +Fair

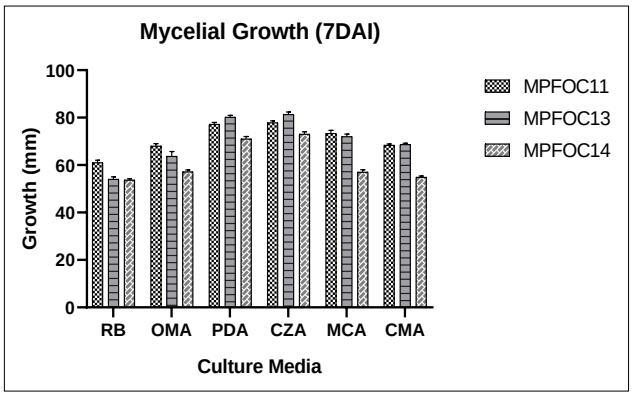


Fig 2 Graphical representation of mycelial growth in different nutritional media

The current research demonstrated the impact of solid medium on the sporulation of *F. oxysporum* f. sp. *ciceri*. All six-culture media evaluated and showed a wide range of sporulation for all the test pathogen, from fair (+) to high (+++). However, high sporulation (+++) was recorded on Potato dextrose agar (PDA) and Czapek's dox agar medium (CZA), whereas Moderate (++) sporulation was observed with Corn meal agar (CMA), Modified Czapek's dox agar (MCA) medium recorded moderate fair (++) sporulation (°C). Findings of Kadam [24] reported that fungus grew best on Czapek's dox agar medium (CZA) followed by Potato dextrose agar medium (PDA), Asthana and Hawker's medium and Snyder and Han's medium. In addition findings were also support of present study which revealed that fungus grew best and sporuled on Potato dextrose agar medium (PDA) and they observed least growth of fungus on Modified Czapek's dox agar [25]. Potato dextrose agar (PDA) is the best medium for the growth and sporulation of different Fusarium isolates [26].

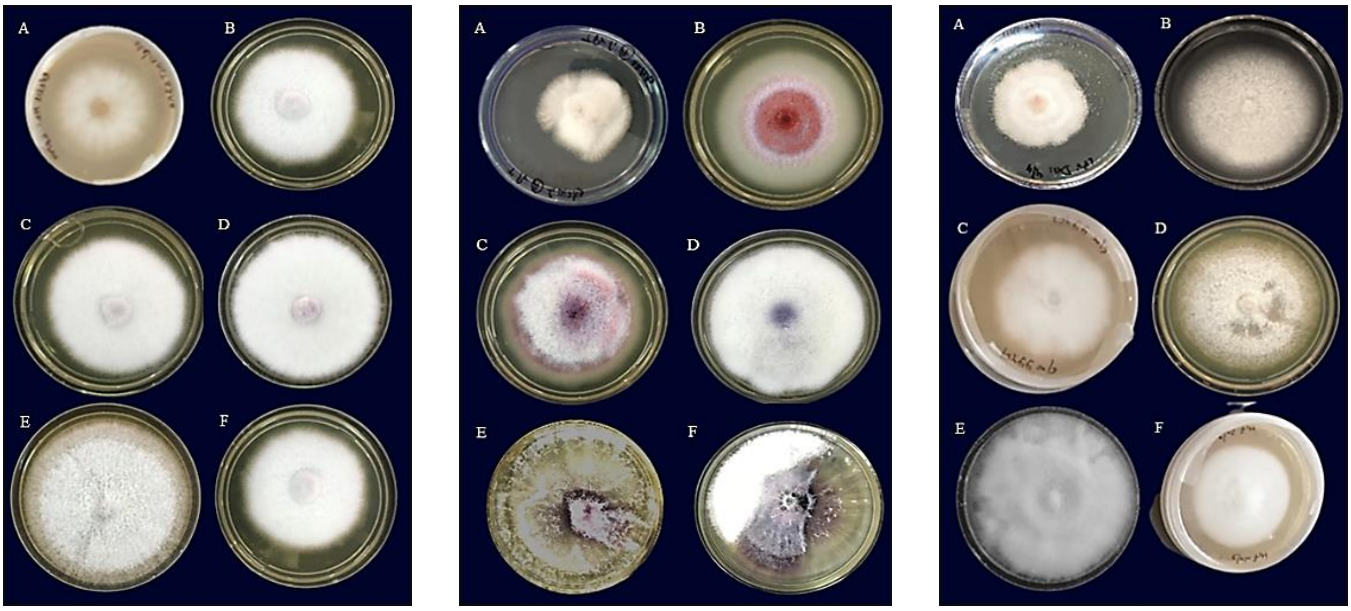
Physiological variability

The three FOC isolates were assessed for their mycelial growth in different physiological condition by observing them in different level of temperature and pH level.

Effect of temperature on radial growth and sporulation of FOC isolates

All three FOC isolates grew at the six different temperature range between 10–35 °C to observe their effect on mycelial growth (Fig 3) and sporulation after 7 days of incubation. Observation revealed that the maximum radial growth was found among 25 °C and 30 °C. Highest colony mean diameter was obtained in MPFOC11 (75.00 mm) and

MPFOC13 (71.66 mm) at 30 °C whereas the lowest one was 21.16 mm and 21.33 mm which was observed at 10 °C and 35 °C in MPFOC14 respectively (Table 5, Fig 4). Findings of Farooq *et al*. [27] are in support of the present findings in which they reported that the growth of the *F. oxysporum* f. sp. *ciceri* was significantly reduced below 15 °C and started to drop above 35 °C, study revealed that high temperatures did not favour the growth of the wilt pathogen. After seven days of inoculation at 25 °C and 30 °C, the fungus got the maximum growth. While at 15 °C, it was reduced significantly. The highest sporulation of micro conidia was observed at 25 °C after seven days of incubation period. They observed minimum sporulation at 15 °C. They also observed that Spore production was not observed at 5 °C. They observed maximum sporulation of macro conidia at 25 °C after seven days of incubation period. The minimum sporulation was observed at 15 °C and at 35 °C.



MPFOC11 MPFOC13 MPFOC14

Fig 3 Mycelial growth of three FOC isolates after seventh day incubation on different temperature
(A) 10 °C; (B) 15 °C; (C) 20 °C; (D) 25 °C; (E) 30 °C; (F) 35 °C

Table 5 Assessment of mycelial growth of three FOC isolates at different temperature

Treatment	MPFOC11		MPFOC13		MPFOC14		Sporulation
	Mean	S.E.	Mean	S.E.	Mean	S.E.	
10	28.167	0.601	27.500	0.500	21.333	0.333	+
15	35.500	0.289	30.500	0.289	32.333	0.333	++
20	60.500	0.577	63.000	0.289	56.833	0.167	+++
25	62.500	0.577	65.333	0.601	59.500	0.289	++++
30	75.000	0.289	71.667	0.334	64.000	0.289	++++
35	23.417	0.795	22.500	0.289	21.167	0.441	++
C.D.	1.719		1.254		0.994		
SE(m)	0.552		0.403		0.319		
SE(d)	0.780		0.569		0.451		
C.V.	2.011		1.491		1.300		

++++High; +++Moderate; ++Semi-moderate; +low

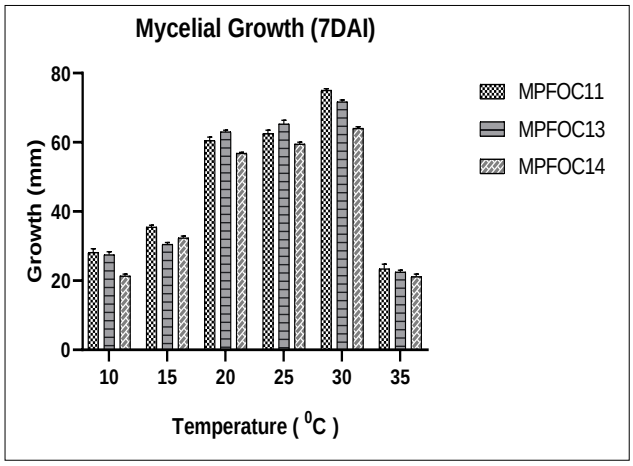


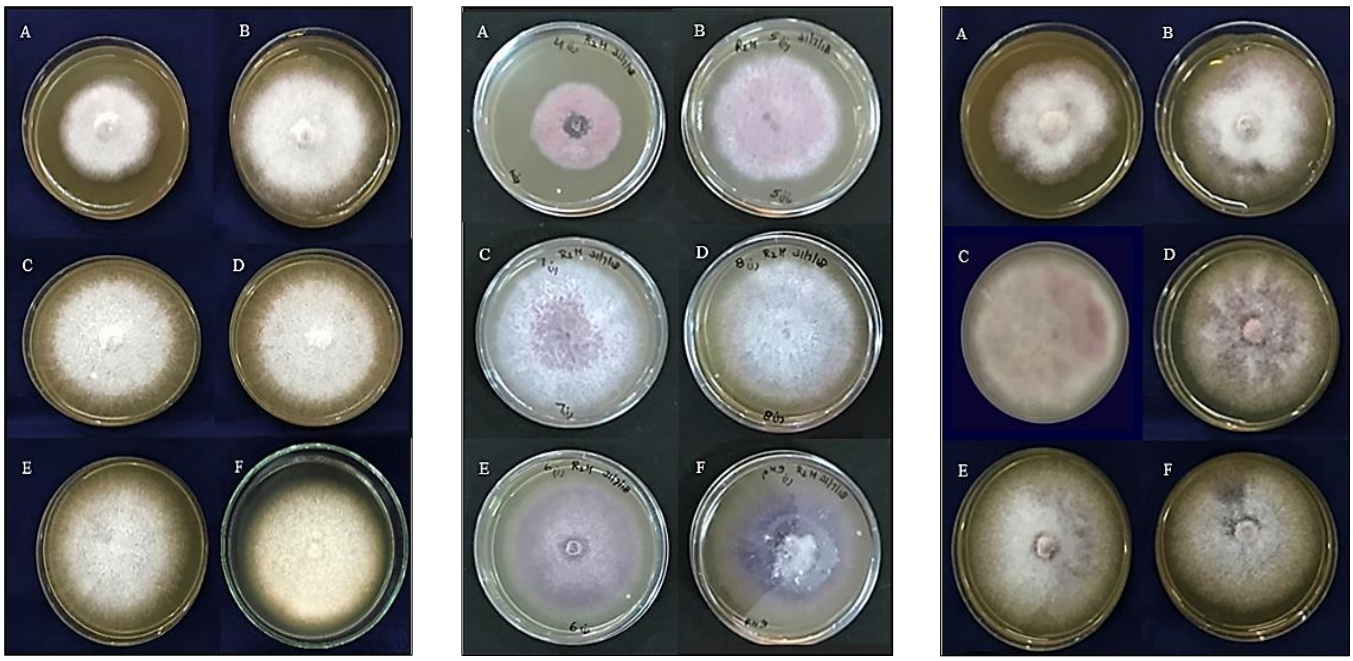
Fig 4 Graphical representation of mycelial growth at different temperature

Barhate [28] found abundant sporulation after seven days of incubation at 27±2 °C on potato dextrose agar medium, their observation were in support the result obtained from present study. Highest growth of pathogen observed by Nathawat *et al*. [29] 2017 at 30 °C with higher sporulation after seven days of incubation, drastically reduced sporulation below 15 °C and above 35 °C, whereas Chauhan [30]; Desai *et al*. [31], Sharma *et al*. [32] found 25 °C as the optimum temperature for growth of pathogen and Fusarium wilt disease development. However, Mina and Dubey [33] observed maximum colony diameter at 28 °C, whereas 25 °C temperature was also found suitable one for mycelial radial growth and spore production of *Fusarium oxysporum* f. sp. *Cicer*.

Effect of pH on mycelial radial growth and sporulation

In this experiment three FOC isolates were tested at six different pH levels, which exhibited a wide range of sporulation from low (+) to high (++++). The findings of this experiment

indicated that at pH 6.0 and pH 7 all of the three FOC isolates exhibited good radial growth (Fig 5). The highest colony diameter was noted for the isolate MPFOC13 (83.00 mm) followed by MP FOC-14 (82.00 mm) at pH 6.0, at pH 4 the lowest mycelial radial growth in isolate MPFOC13 (52.00 mm), MPFOC14 (52.66) and MPFOC11 (53.00) was observed thrice of which at par. Sporulation rate was very low at pH 4, pH 5, pH 8 and pH 9 however at pH 6 and pH 7 it was high (Table 6, Fig 6). Farooq *et al*. [27] also observed that *Fusarium oxysporum* f. sp. *ciceri* grow well at pH 7 after seven days of inoculation, they also observed that the growth of the fungus reduced by wavering the pH level from the neutral level. Optimum pH for the growth of *Fusarium oxysporum* f. sp. *ciceri* ranged from pH 6.5 to 7.0. *Fusarium oxysporum* f. sp. *ciceri* has capacity to stand at a wide range of pH i.e., 5.0–6.5 [34]. After seven days of incubation period, they observed maximum micro conidia production at pH 6.0 and minimum sporulation at pH 4.5 as well as maximum macro conidia were produced at pH 6.0 and minimum sporulation or no macro conidia was observed at pH 4.5. Khilare and Ahmed [35] reported highest sporulation of *Fusarium oxysporum* f. sp. *ciceri* at pH 6.0.



MPFOC11 MPFOC13 MPFOC14

Fig 5 Mycelial growth of three FOC isolates after seventh day incubation on different pH
(A) pH 4; (B) pH 5; (C) pH 6; (D) pH 7; (E) pH 8; (F) pH 9

Table 6 Assessment of mycelial growth of three FOC isolates at different pH

Treatment	MPFOC11		MPFOC13		MPFOC14		Sporulation rate
	Mean	S.E.	Mean	S.E.	Mean	S.E.	
pH 4	53.000	0.577	52.000	0.577	52.667	0.333	+
pH 5	67.000	0.577	68.000	0.577	67.000	0.577	++
pH 6	79.000	0.577	83.000	0.577	82.000	0.577	+++
pH 7	77.000	0.577	80.667	0.334	77.000	0.577	+++
pH 8	70.333	0.333	73.000	0.577	72.667	0.334	++
pH 9	66.333	0.333	68.000	0.577	65.333	0.333	++
C.D.	1.586		1.696		1.469		
SE(m)	0.509		0.544		0.471		
SE(d)	0.720		0.770		0.667		
C.V.	1.282		1.332		1.176		

+++High; ++moderate; +low

CONCLUSION

In future breeders could use the considerable information obtained by the current study to develop resistant varieties of chickpea that are particular to a given geographic area in terms of morphological and pathogenic diversity of *F. oxysporum* f sp *ciceri*. Knowledge of the fungal physiology in different growth conditions as well as information about cultivate habits could be helpful for future research about fusarium wilt. A deeper understanding of in vivo culture growth characteristics could be a crucial for future research.

Conflict of interest

The authors asserted no conflict of interest. This research received no external funding.

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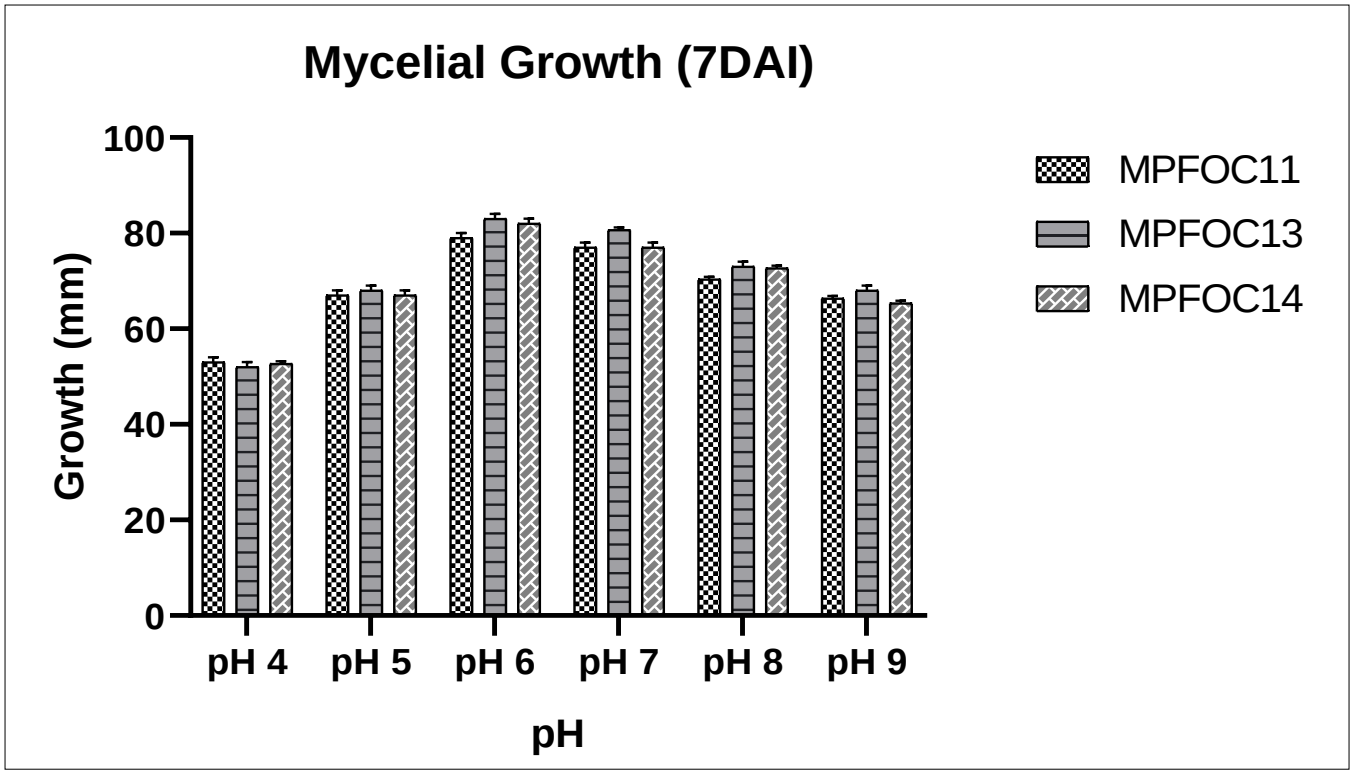

Fig 6 Graphical representation of mycelial growth in different pH

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