Original Research Article

Identifying *F. oxysporum* Strains Causing Wilt in Southern Indian Chickpeas

ABSTRACT

Fusariumoxysporum(Schletend: Fr) f. sp. *ciceri* (Padwick), referred to as Foc, is a soil-borne fungus that poses a constant threat to chickpeas (*Cicer arietinum* L.) by causing wilt disease. Typical wilt symptoms were observed in chickpea plants collected from 24 different locations across three southern Indian states. The process involved isolating *Fusarium* species from the roots of these wilted plants, resulting in the identification of various strains exhibiting diverse cultural and morphological characteristics on potato dextrose agar medium. All twenty four isolates were subjected to Koch's postulates using the standard method, which yielded varied responses in terms of disease incidence. After analyzing cultural, morphological, molecular traits, and conducting pathogenicity tests, the fungus was definitively identified as *F. oxysporum* Schlechtend. Fr. f. sp. *ciceri*(Padwick) Matuo and K. Sato. Among the 24 isolates tested on the chickpea wilt susceptible cultivar JG-62, one was non-pathogenic with zero percent disease incidence (PDI), while one isolate was highly pathogenic ~~isolate~~showed 100 percent PDI. Highly pathogenic four isolate was further used for molecular identification with secreted in xylem primers (*SIX*). Comparative studies of cultural traits and conidial morphology among different isolates revealed variations in growth patterns, pigmentation, sporulation, and the size and structure of macro and micro conidia, as well as chlamydospores.

Keywords: Fusariumwilt, chickpeas, disease, strains, cultivar, morphology, disease incidence.

1. INTRODUCTION

Chickpea (*Cicer arietinum* L.) holds the position of the fourth most significant legume crop globally, following soybean, common bean, and peas(Please add Reference). In less developed nations, chickpeas play a vital role in enhancing cereal-based diets due to their substantial nutritional benefits. Primarily cultivated for its protein-rich edible seeds, this crop serves purposes in both seed and forage production (1).Worldwide, chickpeas are farmed across approximately 14.56 million hectares, resulting in a total output of around 14.78 million tons, with an average yield of about 1014.60 kilograms per hectare(Please add Reference). In the specific case of India, chickpeas are cultivated over an expanse of 15 million hectares, leading to a production of roughly

15.87 million tons and an average yield of approximately 1058 kilograms per hectare (data sources: FAOSTAT, 2021; Agricultural Statistics at a Glance, 2021).

Fusarium wilt, resulting from the fungus *Fusariumoxysporum* Schlechtend. f. sp. *ciceri* (Padwick) Matuo& K. Sato, (Foc) is a significant fungal pathogen widely spread in chickpea cultivation regions globally. It has been documented in approximately 33 countries (2). The occurrence of *Fusarium* wilt leads to substantial annual reductions in chickpea yields, comprising about 10 to 15 percent of the total production. In certain conditions conducive to the disease, the losses can even escalate to 100 percent (3). Estimates for crop losses indicate roughly 10-15 percent annually, with more severe epidemics causing losses as high as 60-70 percent (4).

Fusariumoxysporum f. sp. *ciceri*(Foc) displays considerable variability as a pathogen. There have been reports of eight distinct races of this pathogen, with six (1A, 2, 3, 4, 5, and 6) inducing symptoms of wilting(5). Within India, four races of FOC(please follow a uniform pattern either Foc or FOC) (1A, 2, 3, and 4) are common, and among them, race 1A exhibits the highest level of virulence and severe epidemics causing losses as high as 60-70 percent (4).

Presently, the research aimed to improve efforts in breeding for higher chickpea yield and production by developing chickpea cultivars that possess resistance against wilt. The primary goals included accurately and swiftly identifying and diagnosing the pathogen.

(Please add necessary inputs regarding importance of the study for Southern India).

2. MATERIALS AND METHODS

2.1 Sample collection, isolation and purification

Chickpea plants exhibiting characteristic wilting symptoms were gathered from twenty-four distinct locations across Southern Indian states, including Nizambad, Mahabubnagar, Rangareddy, Medak, Kurnool, Nandyal, Anantpur, Dharwad, Raichur, Bijapur, Kalaburgi, and Bidar, encompassing the regions of Telangana, Andhra Pradesh, and Karnataka. The fungal organism was isolated from the vascular plant tissue using tissue isolation methods, followed by purification of resulting fungal cultures through single spore isolation techniques. These purified cultures were preserved on PDA slants, stored at 4°C in a refrigerator, and subjected to monthly transfers to sustain the cultures for subsequent investigations. The fungus was isolated, purified, and sub-cultured in a sterile environment within a laminar flow cabinet to ensure aseptic conditions.

The pathogen's isolates were predominantly identified by observing colony characteristics and spore morphology (6). Photomicrographs of the *F. oxysporum* f. sp. *ciceri* isolates were captured using an imaging microscope to depict spore morphology.

(thepictorial evidence of key characters used for identification may be added)

2.2 Demonstrating Koch's principles (Pathogenicity)

The pathogenicity of twenty-four isolates was assessed on the susceptible chickpea wilt cultivar JG-62 during the rabi season of 2021-22 in a controlled glasshouse environment. Each isolate of *Fusariumoxysporum* f. sp. *ciceri* was cultivated on PDB media at 28 ± 2ºC for 10 days to create inoculum. This inoculum was used for root dip inoculation of *Fusarium*. Sets of three pots were prepared for each isolate, with one set containing sterilized soil only for comparison as an uninoculated control(7).

Chickpea seedlings were grown in sand pockets(please mention the period), and transplanted them into main pots and watered as needed, and observed for disease symptoms. Secondary inoculation(at what stage) was performed using inoculum from potato dextrose broth. Except for the control, all pots underwent inoculation. When disease symptoms emerged, the fungus was re-isolated from diseased plant roots and subjected to Koch's postulates to prove its pathogenicity. The percentage of wilt incidence was determined using the provided formula.

$$\text{Per cent Disease Incidence (DI \%)} = \frac{\text{Total number of wilted plants per pot}}{\text{Total number of plants per pot}} \times 100$$

2.3 Characterizing various Foc isolates, for their cultural, morphological, and molecular aspects.

Cultural and morphological studies

Each of the twenty four samples of *F. oxysporum* f. sp. *ciceri* were cultured individually on Potato Dextrose Agar (PDA) in Petri dishes and kept in an incubator at a temperature of 28 ± 2ºC for a duration of seven days. After one week from the initial inoculation, notations were made concerning cultural attributes such as colony appearance, color, growth patterns, and pigmentation.
The spores of various isolates were examined for their morphological traits using stained slides and an imaging microscope.

(Please mention the reference used for key characters or taxonomic studies)

2.4 Molecular characterization

Two isolates(What are the two isolates) that exhibited high virulence(is it high virulence or incidence??, if virulence, please mention the method used for virulence test) in the pathogenicity assessment were identified through the implementation of molecular techniques using the specified protocol.

2.4.1 Fungal DNA isolation and *SIX* (Secreted in xylem)gene region amplification

The genomic DNA of the fungus was obtained from mycelia that were cultivated in 250 ml of PDB at 28 °C for a duration of 4 days. These mycelia were freeze-dried in liquid nitrogen and stored at -80 °C for later use. Genomic DNA was extracted using the CTAB method, as described by Sharma et al. (2014). The Foc specific *SIX* gene region of the fungi, encompassing *SIX* 5 (F) (5'- ATGCTACTAGCTTCGACGGGATTG -3'), and *SIX* 5 (R) (5'-TTACTCCGTGCATTGAATGTACC -3'), was amplified. The amplification process occurred in a 50 µl reaction mixture with 100 pmol of both forward and reverse primers. A PCR reaction was used with an initial denaturation step at 94 °C for 4 minutes, followed by 35 cycles at 94 °C for 1 minute, 57 °C for 1 minute, and 72 °C for 1 minute. The final extension was performed at 72 °C for 5 minutes. Afterwards, DNA and RNA bands on an agarose gel were visualized under ultraviolet (UV) light once the gel was stained with a fluorescent dye like ethidium bromide and illuminated with UV light.

(Please mention the reference article for molecular detection)

3. RESULTS AND DISCUSSION

3.1 The pathogen Isolation and purification of pathogen

The chickpea plants suffering from wilt were recognized in the field by prominent indications such as drooping, leaf yellowing, and plant desiccation. The roots of these affected plants, when vertically split, exhibited a brown staining of the xylem vessels (**Fig**. 1A). The causative agent responsible for the wilt was obtained from the afflicted plants using the tissue segment technique on PDA. The fungus was subsequently purified using the single spore isolation method on PDA. The pure culture was showed in **Fig**ure 2**.**Sharma et al. (2014) employed a comparable approach to isolate the pathogen from chickpea plants afflicted with wilt.

Fig. 1.A) Typical Fusarium wilt symptoms exhibited in the field during seedling and adult plant stages(Field photograph shall be more explanatory covering at least a few portion of the field and not single plant). B) JG 62 seedlings were grown in sand pockets C) Pathogenicity of Foc isolates in glasshouse(Closer shots / photos of symptom is needed)

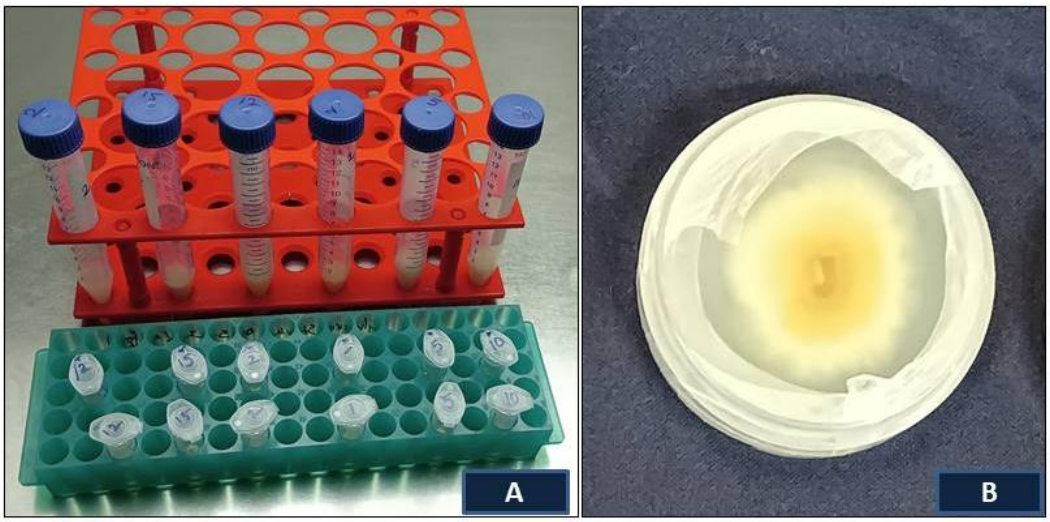

Fig. 2.A) Single spore isolation by serial dilution B) Pure culture of Foc

3.2 Pathogenicity of isolates

The pathogenicity of twenty-four isolates of *Fusariumoxysporum* f. sp. *ciceri* was evaluated using the "Root Dip Inoculation method" on the susceptible chickpea wilt cultivar JG-62, as outlined in the "Materials and Methods" section (**Fig**. 1B and IC). The results of the pathogenicity test revealed variability among these isolates in terms of the extent of infection(4).

Among all the tested isolates, the Foc 14 isolate exhibited the highest disease incidence at 100%, while the Foc 3, 10, 13, 21, 22 isolates had disease incidences of more than 87.5% each. The other Foc isolates displayed a more than 25% disease incidence. On the other hand, the Foc 5 isolate was deemed non-pathogenic shown in Table 1.

(The table (1) shall be re-aligned at the right place appropriately)

This outcome suggests that the various strains of fungi obtained from the affected roots might possess varying levels of pathogenicity. Therefore, it is necessary to subject the received isolate(s) in a pure culture to additional testing to determine their pathogenic nature. This will allow the selection of the pathogenic culture for subsequent laboratory and field experiments.

Our findings aligned with those ofPandey et al. (2012)(8), who validated the pathogenic nature of *Fusariumoxysporum* f. sp. *ciceri* through the use of the Root dip inoculation method in earthen pots within a greenhouse setting, employing the susceptible cultivar JG-62.

3.3 Identification of the pathogen

3.3.1 Cultural identification

Cultural traits of *Fusariumoxysporum* f. sp. *ciceri*, including colony color, growth, pigmentation, and sporulation, were documented one week after inoculation and are displayed in Table 2**.**

The distinct (please recheck the table 2, as the characters are not distinct – red bold - 1 and 4)cultural traits of 24 Foc isolates showed variations in colony appearance, growth behavior, pigmentation, and spore production. A majority of the isolates exhibited a pale white to typical cottony white colony color. Furthermore, the isolates differed in their mycelial arrangement and growth pattern, as illustrated in **Fig**ure 3. Based on their mycelial growth, the isolates were grouped into two categories: sparse growth and dense growth. While most isolates displayed dense or sparse growth with smooth margins.The majority of the isolates displayed the usual pale yellow coloring even after a month of incubation. However, two isolates, namely Foc 3 and 9, exhibited a brown pigmentation, while the Foc 15 isolate had a light brown pigmentation.

Foc 3, 10, 13, 14 21 and 22 isolates exhibited strong sporulation. Six isolates displayed moderate sporulation: Foc 1, 2, 4, 6, 7, 8, 9, 11, 12, 15, 16, 17, 19, 20 and 23. Foc 5, 9, 18, 24 isolates, on the other hand, demonstrated low sporulation.

The observations indicate that there is a connection between the virulence of the isolates and their sporulation. Isolates that displayed high levels of sporulation were found to be highly virulent, whereas isolates with low to moderate sporulation showed very low levels of pathogenicity or were non-pathogenic.

Based on their growth patterns, the isolates were divided into three groups: rapid growth, moderate growth, and slow growth. Among them, Foc 5, 6, 10, 11, 13, 14, 17, 23, 24 isolates demonstrated fast growth, while Foc 2, 8, , 18, 19 and 22 isolates exhibited slow growth. The remaining isolates displayed a moderate level of growth. In this study, highly virulent isolates shared common characteristics, including varying degrees of mycelial density, pale yellow pigmentation, moderate to fast growth, and abundant sporulation(the common characters shall be precise).

Table. 1 Displays the differences in wilt disease incidence among various strains of *Fusariumoxysporum* f. sp. *ciceri*.

S. No.	Name of the State	Name of the Mandal	Foc designation	Total no. of plants	Total wilted plants	Percent disease incidence
1	Telangana	Bhainsa	Foc 1	15	12	80

2	Telangana	Kolur	Foc 2	15	11	73.5
3	Telangana	Arepalli	Foc 3	15	13	87
4	Telangana	Mugpal	Foc 4	15	12	80
5	Telangana	Alampur	Foc 5	15	0	0
6	Telangana	Undavelli	Foc 6	15	8	53.5
7	Telangana	Yelwarthy	Foc 7	15	9	60
8	Telangana	Nyalata	Foc 8	15	11	80
9	Telangana	Kadloor	Foc 9	15	14	3.5
10	Telangana	Peddapur	Foc 10	15	13	87
11	Andhra Pradesh	Narayanapuram	Foc 11	15	8	53.5
12	Andhra Pradesh	Pusaluru	Foc 12	15	7	47
13	Andhra Pradesh	Gunthakal	Foc 13	15	13	87
14	Andhra Pradesh	Nandyal	Foc 14	15	15	100
15	Karnataka	Bayahati	Foc 15	15	12	80
16	Karnataka	IIPR Dharwad	Foc 16	15	8	53.5
17	Karnataka	Ashikar	Foc 17	15	9	60
18	Karnataka	Masavakal	Foc 18	15	4	27
19	Karnataka	MahalTanda	Foc 19	15	7	47
20	Karnataka	Byrudigi	Foc 20	15	11	73.5
21	Karnataka	KVK, Kalaburgi	Foc 21	15	14	93.5
22	Karnataka	Hasargundgi	Foc 22	15	13	87
23	Karnataka	Markhal	Foc 23	15	12	80
24	Karnataka	Kallur	Foc 24	15	5	33.5

Table.2 Distinctive traits exhibited by various strains of *Fusarium oxysporum* f. sp. *ciceri* in colonies

S. No.	Foc designation	Mycelial arrangment and color	Pigmentation	Growth habit	Sporulation*
1	**Foc 1**	**Dense Cottony white**	**Pale yellow**	**Moderate**	**+++**
2	Foc 2	Sparse Cottony white	Pale yellow	Slow	+++
3	Foc 3	Dense Dirty white	Brown	Moderate	+++
4	**Foc 4**	**Dense Cottony**	**Pale yellow**	**Moderate**	**+++**

		white			
5	Foc 5	Dense Cottony white	Pale yellow	Fast	+
6	Foc 6	Sparse Cottony white	Pale yellow	Fast	++
7	Foc 7	Sparse Cottony white	Pale yellow	Moderate	+++
8	Foc 8	Dense Cottony white	Pale yellow	Slow	+++
9	Foc 9	Dense Cottony white	Brown	Slow	+
10	Foc 10	Dense Dirty white	Pale yellow	Fast	+++
11	Foc 11	Sparse Cottony white	Pale yellow	Fast	++
12	Foc 12	Dense Cottony white	Pale yellow	Moderate	++
13	Foc 13	Dense Dirty white	Pale yellow	Fast	+++
14	Foc 14	Sparse Cottony white	Pale yellow	Fast	++++
15	Foc 15	Dense Cottony white	Light Brown	Moderate	+++
16	Foc 16	Dense Dirty white	Pale yellow	Moderate	++
17	Foc 17	Dense Cottony white	Pale yellow	Fast	+++
18	Foc 18	Dense Cottony white	Pale yellow	Slow	+
19	Foc 19	Sparse Cottony white	Pale yellow	Slow	++
20	Foc 20	Sparse Cottony white	Pale yellow	Moderate	+++
21	Foc 21	Dense Dirty white	Pale yellow	Moderate	+++
22	Foc 22	Dense Dirty white	Pale yellow	Slow	+++
23	Foc 23	Sparse Cottony white	Pale yellow	Fast	+++
24	Foc 24	Sparse Cottony white	Pale yellow	Fast	+

* + Poor, ++ Moderate, +++ Profuse, ++++ Abundant

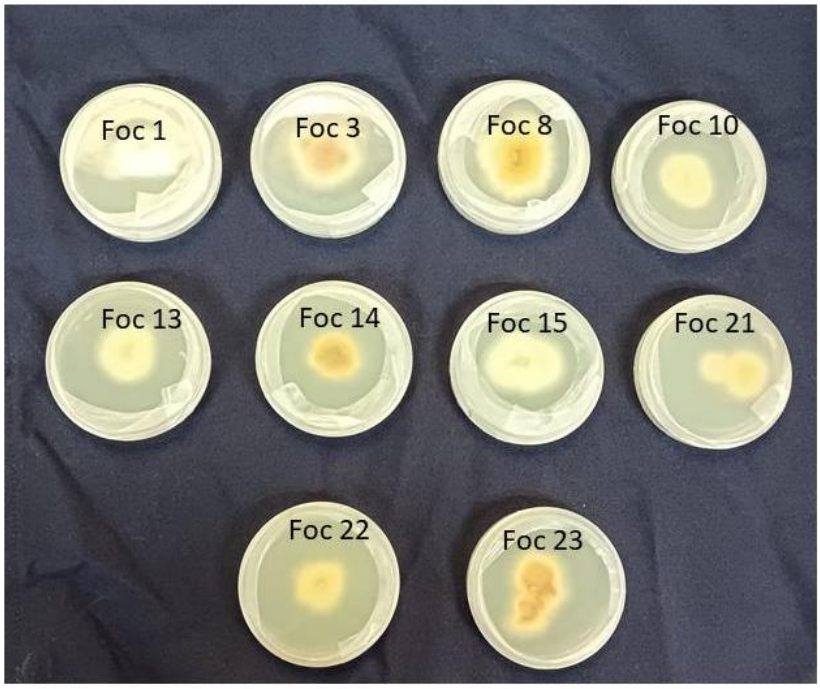

Fig.3Distinctive cultural traits exhibited by various isolates of *Fusarium oxysporum* f. sp. *ciceri*.(Please include the remaining plates)

Several researchers including Prasad and Padwick (1939), (8, 9, 10, 11, 12, 13)have investigated chickpea wilt and identified variations in pathogenicity among F. *oxysporum* f. sp. *ciceri* isolates. Paulkar and Raut (2004)(14) also observed differences in mycelial growth patterns. Different pigmentation such as brownish, light yellow, and violet within the isolates has been documented by various researchers (10, 15, 16, 17)

Honnareddy and Dubey (2007) (18)discovered differences concerning colony color, substrate pigmentation, growth rate, presence of macro conidia, and virulence on the susceptible variety L 550. Correspondingly, *Fusarium* wilt isolates' colony growth, size, and pigmentation were found to be highly variable, as noted by Dubey et al. (2010), Mandhare et al. (2011) (18, 19). This aligns with the findings of the present investigation. Similarly, Singh et al. (2010)(20) observed variations in growth characteristics, ranging from dull white to pinkish white, with thin to fluffy hairy growth and irregular margins.

3.3.2 Morphological identification

The fungus *Fusariumoxysporum* f. sp. *ciceri* produces two types of conidia: microconidia, which are small in size, and macroconidia, which are larger. The dimensions of the conidia, including width and length, were measured for 24 different isolates. These measurements are visualized in **Fig**ure 4A and 4B. Upon microscopic examination, it was observed that the microconidia (shown in **Fig**. 4B) across all isolates were small(means morphologically the isolates are not different??, please

substantiate), consisting of one to two cells. They appeared hyaline and exhibited oval to reniform shapes, with some being oval to oblong and slightly curved. Their lengths ranged from 8.45 to 17.10 µm, while their widths varied from 2.49 to 4.72 µm. Notably, there was significant variability in the size of microconidia among the isolates(please be specific to isolates and not generalize).

The macroconidia present in all these isolates exhibited elongated and diverse forms, maintaining a fairly consistent width except at the tip where they curved, becoming narrower and terminating in a smoothly rounded or pointed end. They were mostly composed of 2-3 septa, and appeared translucent. Their lengths varied between 15.05 and 23.09 µm, with widths ranging from 3.75 to 5.89 µm(please be specific to isolates and not generalize).

In old cultures, chlamydospores were formed. These spores had either a rough or smooth outer surface, and they could be positioned in the middle or at the end of the structure. They had the potential to form individually, in pairs, or in chains (as shown in **Fig**ure 4B) (please be specific to isolates and not generalize).

The comparison between the size and septation of macro and micro conidia, as well as chlamydospores, in both pathogenic and non-pathogenic strains did not provide a clear understanding. The current study highlights that measuring conidia does not correlate with their virulence, which aligns with findings from Patil et al. (2005)(7) showing variations in F. *oxysporum* f. sp. *ciceri* isolates regarding conidia number, size, cultural traits, growth pattern, pigmentation, and sporulation. Similarly, Dubey et al. (2010) (18)noted microconidia sizes ranging from 5.1-12.8 x 2.5-5.0 µm and macroconidia from 16.5-37.9 x 4.0-5.9 µm with 1-5 septations. Gupta et al. (1986) (10)reported microconidia sizes of 3.88-9.99 x 1.66-4.99 µm and macroconidia sizes of 16.65-66.60 x 3.33-6.66 µm. The present study similarly observed these conidia dimensions among different F. *oxysporum* f. sp. *ciceri* isolates.

3.3.3 Molecular Identification

(The necessity for molecular identification may further be elaborated. What was the reason to select only high pathogenic isolates, which was a laboratory study??)

Highly pathogenic four isolates underwent molecular characterization using Foc-specific *SIX* primers, resulting in successful amplification for each isolate (see **Fig**. 5).

(Foc 14 isolate exhibited the highest disease incidence at 100%, while the Foc 3, 10, 13, 21, 22 isolates exhibited 87.5% each, that means there are six isolates with high pathogenic efficiency, why only 3, 10, 14 and 21 were choosen??)

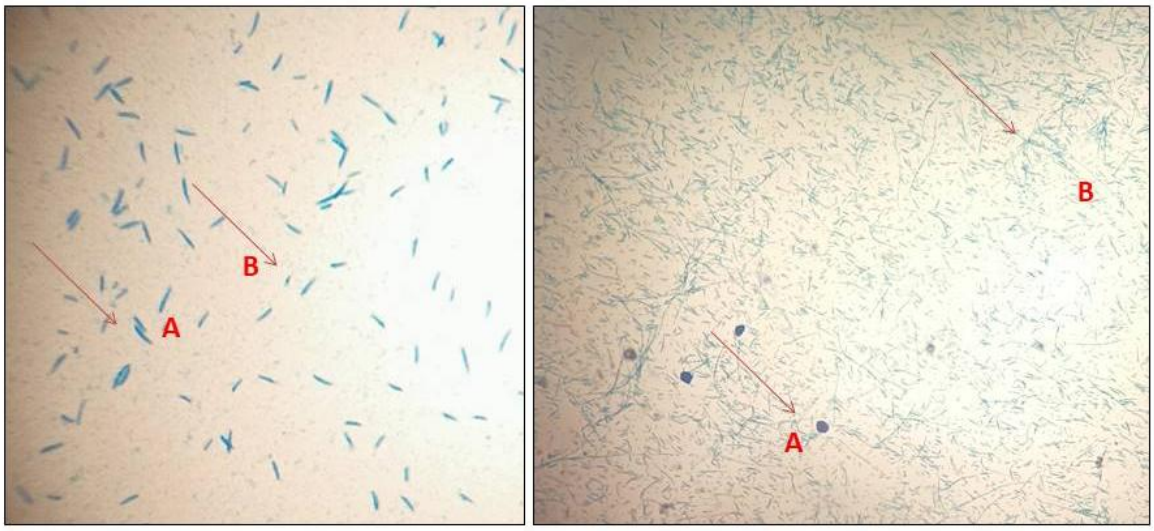

Fig. 4(a).A) Macroconodia B) Microconidia**Fig**. 4(b). A) Chlamydospore B) Mycelium(Please re-align the pictures appropriately and add pics with respect to isolates)

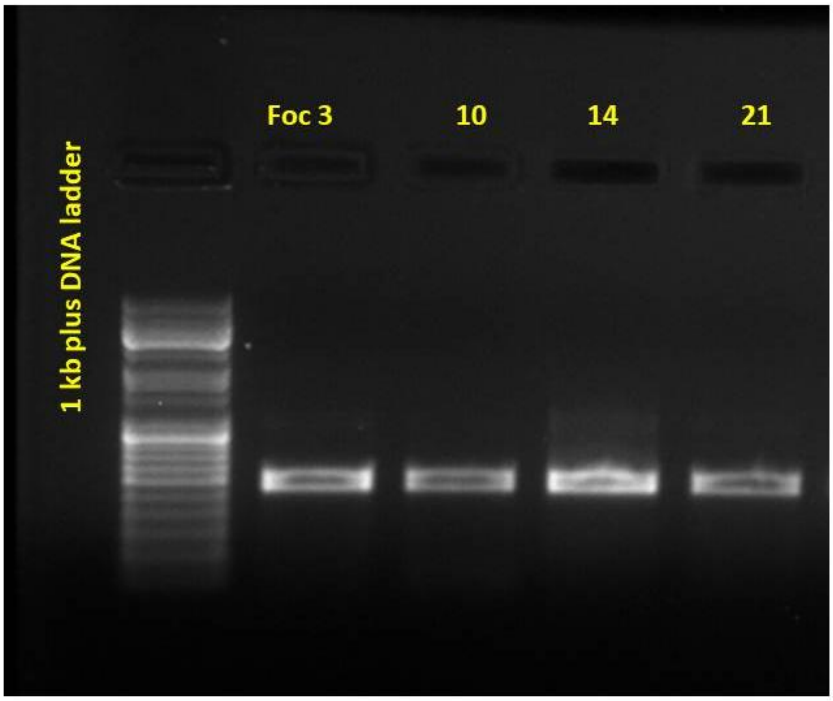

Fig. 5 Amplification of four Foc isolates with Foc specific *SIX* geneprimers(please mention the ladder size and size of the amplified product)

(please include the work done in reference to relevant literature and compare elaborately for molecular studies)

4. CONCLUSION

The soil-borne fungus *Fusarium oxysporum* f. sp. *ciceri*(Foc) poses a constant threat to chickpeas in southern India, causing wilt disease. Wilt symptoms were observed in chickpea plants from 24 locations. Various Fusarium strains were isolated from wilted plant roots, showing diverse characteristics on agar medium. Koch's postulates were used to test 24 isolates, resulting in different disease responses. The fungus was identified as F. oxysporum Schlechtend. Fr. f. sp. ciceri after cultural, morphological, and molecular analysis. Among 24 isolates, one was non-pathogenic, while another highly pathogenic one showed 100% disease incidence. Comparisons revealed variations in growth, pigmentation, sporulation, and conidial traits among isolates.

(Conclusion shall be more narrative and avoid the same as abstract)

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