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ITS rDNA Analyses in the Identification and Differentiation of Isolates of *Fusarium oxysporum* f. sp. *ciceri* Causing Chickpea Wilt

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A B S T R A C T

Eight variants of *Fusarium oxysporum* f. sp. *ciceri* were isolated from wilt infected chickpea plants from diverse locations of northern Karnataka. Genetic variability was studied by polymerase chain reaction (PCR) amplification with specific primer and genetic identity among each variant was calculated and results depicted that there was minimum genetic variation among the variants collected from the diverse locations. At 0.01 similarity coefficient the variants separated into two clusters. Cluster A contained six variants *viz.*, Foc V, Foc Ku, Foc B, Foc S, Foc Ka and Foc H and cluster B contained two variants Foc R and Foc J.

Introduction

Chickpea (*Cicer arietinum L.*) is one of the most economical and oldest pulse crop after beans and peas. Chickpea seeds contain an average of 23 per cent protein, 38-59 per cent carbohydrate, 4.8-5.5 per cent oil, 47 per cent starch, 5 per cent fat, 6 per cent crude fibre, 6 per cent soluble sugar and 3 per cent ash, minerals such as calcium (202 mg), phosphorous (312 mg), iron (10.2 mg), vitamin C (3.0 mg), calorific value (360 cal), small amounts of B complex, fibre (3.9 g) and moisture (9.8 g) (Singh, 1985).

Chickpea is infected by 172 pathogens (67 fungi, 3 bacteria, 80 nematodes, 22 viruses and phytoplasma) across the universe (Nene *et al.*, 1996). Out of all, only a few are having potential to devastate chickpea. Some of the dangerous diseases in order of their importance are wilt, dry root rot, collar rot, colletotrichum blight, alternaria blight, rust and ascochyta blight caused by *Fusarium oxysporum* f. sp. *ciceri*, *Macrophomina phaseolina*, *Sclerotium rolfsii*, *Colletotrichum dematium*, *Alternaria alternata*, *Uromyces ciceris-arietini* and *Ascochyta rabiei* respectively (Nene *et al.*, 1984).

Yield loss in chickpea from Fusarium wilt have been varied from 10 to 15 per cent (Jalali and Chand., 1991; Trapero-Casas and Jimenez-Diaz., 1985) but damage up to 70 per cent have been recorded in some years in Northern India and Pakistan (Grewal and Pal.,

1970). As a facultative saprophyte, *Fusarium oxysporum* f. sp. *ciceri* can survive in soil and on crop residues as chlamydospores for upto six years.

DNA markers have become a powerful tool to study taxonomy and molecular genetics of a variety of organisms. Since *Fusarium* has a high diversity nature identification of the pathogenic fungi by morphological traits is difficult due to its high variability characters like mycelial pigmentation, formation, shape and size of conidia which are unstable and highly dependent on the composition of media and environmental condition. Thus it leads to the identification of the *Fusarium* by genetic characterization of pathogens which can help in resistance breeding as an effective strategy for management of wilt diseases which can help in understanding the molecular basis of pathogenesis and the resistance mechanism required for effective management strategy.

Materials and Methods

Molecular variability of *Fusarium oxysporum* f. sp. *ciceri*

Fungal variants

Eight variants of *Fusarium oxysporum* f. sp. *ciceri* were collected from infected chickpea plants from four districts of Northern Karnataka, India (Table 1). The variants collected were identified, purified and preserved in PDA medium and confirmation of variants by Koch`s postulation and based on the morphological characters described by Booth (1971).

Isolation of genomic DNA from *Fusarium oxysporum* f. sp. *ciceri*

DNA extraction was done by following standard CTAB method with certain modifications (Patil, 2009). The fungal mycelial mat was crushed finely with pestle and mortar in liquid nitrogen. Then finely crushed fungal mycelial mat is taken in to the Eppendorf tube and 1 ml of extraction buffer was added. Add 10 µl 2-mercaptoethanol and equal volume of phenol: Chloroform: Isoamyl alcohol (1:1 W/V) to the Eppendorf tube and centrifuged at 10,000 rpm for 15-20 minutes at 4ºC.

Supernatant was taken into the new Eppendorf tube and 2.5 µl RNase and 2.5 µl protienase-k was added to the tube. Cooled isopropanol of about 1/3rd volume (300-400 µl) was added. Centrifuged @ 10,000 rpm for 15 minutes at 4ºC. Wash buffer about 500 µl was added and centrifuged at 10,000 rpm for 5 min at 4ºC.

The DNA, pellet was cleaned with 70 % ethanol, vacuum dried and 500 µl of $T_{10}E_1$ is added into the tube. This DNA obtained was further quantified by 0.8 per cent agarose gel electrophoresis. The quantification was done by spectrophotometer and stored at -20$^{°C}$ until further use. The quantity of DNA was determined by monitoring the absorbance at 260 nm in a biophotometer. The A260/A280 ratio was checked for quality of DNA.

Polymerase chain reaction (PCR)

The ribosomal DNA (rDNA) unit contains genetic and non-genetic or spacer region. Each repeat unit consists of copy of 18S, 5.83S and 28S like rDNA and its spacer like internal transcribed spacer (ITS). The rDNA have been employed to analyse evolutionary events because it is highly conserved, whereas ITS rDNA is more variable hence, it was used for investigation.

The primers for amplification were synthesized at Chromous Agri Biotech Pvt Ltd. Bangalore and supplied as lyophilized products of desalted oligos. Primer sequences used are given below.

Other than template DNA the master mix was added to PCR tubes (18 µl/tube) and then 2 µl of template DNA from the respective variants was added to make final volume of 20 µl.

Results and Discussion

Isolation of genomic DNA was made by CTAB method. Thus obtained genomic DNA was observed by running on 1 per cent Agarose gel electrophoresis. The yield of DNA was sufficient for the analysis. The ITS rDNA region was amplified with ITS-1 (5'-TCC GTAGGTGAACCTGCG-3') and ITS-4 (5'-TCCTCCGCTTATTGATATGC-3') primers for variants of *Fusarium oxysporum* f. sp. *ciceri*. DNA amplicon was observed at the region 560 base pairs. The amplified products were checked on 3 per cent Agarose gel electrophoresis.

The DNA sequences were obtained for ITS rDNA. The DNA sequence of eight variants was compared with NCBI blast. The similarity coefficient among eight variants was up to 0.01. These variants of *Fusarium oxysporum* f. sp. *ciceri* was used as an out group vice versa to interpret the clustering of variants as distinct or related out group of genus. In cluster-I (Hulkoti, Kalburgi, Shirur, Bagalkote, Kudgi and Vijayapura), cluster-II (Ron and Jewargi) were grouped (Fig. 1).

Table.1 Designation of the variants of *Fusarium oxysporum* f. sp. *ciceri* the causal agent of chickpea wilt from four districts of Karnataka

Sl. No	District	Location	Variants identified	Variants designation
1	Vijayapura	RARS Vijayapura	*Fusarium oxysporum* f. sp. *ciceri*	Foc V
		Kudgi	*Fusarium oxysporum* f. sp. *ciceri*	Foc Ku
2	Bagalkote	KVK Bagalkote	*Fusarium oxysporum* f. sp. *ciceri*	Foc B
		Shirur	*Fusarium oxysporum* f. sp. *ciceri*	Foc S
3	Gadag	Hulkoti	*Fusarium oxysporum* f. sp. *ciceri*	Foc H
		Ron	*Fusarium oxysporum* f. sp. *ciceri*	Foc R
4	Kalburgi	Kalburgi	*Fusarium oxysporum* f. sp. *ciceri*	Foc Ka
		Jevargi	*Fusarium oxysporum* f. sp. *ciceri*	Foc J

Details of the primers used in the experiment

Organism	Primer code	Sequence
Universal fungus ITS	ITS-1 – f	5'-TCCGTAGGTGAACCTGCG-3'
	ITS-4 – r	5'-TCCTCCGCTTATTGATATGC-3'
Fusarium oxysporum* f. sp. *ciceri	FOC-Fs4-f	5'ATCGGCCACGTCGACTCT$_3$'
	FOC-Fs4r	5'GGCGTCTGTTGATTGTTAGC3'

PCR reaction mixture

Reaction mixture	Quantity
Template DNA (25 ng/µl)	2.00 µl
Primer (5PM/µl)	F-1.00 µl
	R-1.00 µl
dNTPs mix (2.5 mM each)	1.00 µl
15 mM $MgCl_2$	2.00 µl
Taq DNA polymerase (6.0U µl-1)	0.50 µl
Sterile distilled water	12.50 µl
Total	20.00 µl

PCR condition for ITS region, *Fusarium oxysporum* f. sp. *ciceri*

Step	Universal ITS		*Fusarium oxysporum* f. sp. *ciceri*	
	Temperature (°C)	Time period (min)	Temperature (°C)	Time period (min)
Initial denaturation	94	5	94	5
Denaturation	94	1	94	1
Annealing	54	1	58	1
Extension	72	2	72	2
Final extension	72	10	72	20
Hold	4	20	4	20
No. of cycles Denaturation Annealing Extension	35		40	

Fig.1 Phylogenetic relationship based on ITS rDNA among the variants of *Fusarium oxysporum* f. sp. *ciceri* from diverse regions of northern Karnataka

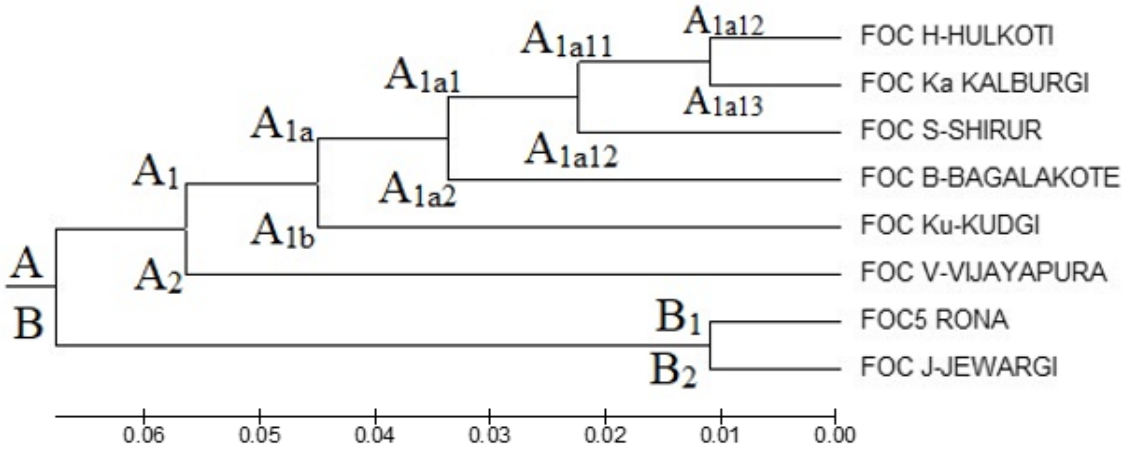


Plate.1a Amplification of ITS region of *Fusarium oxysporum* f. sp. *ciceri*

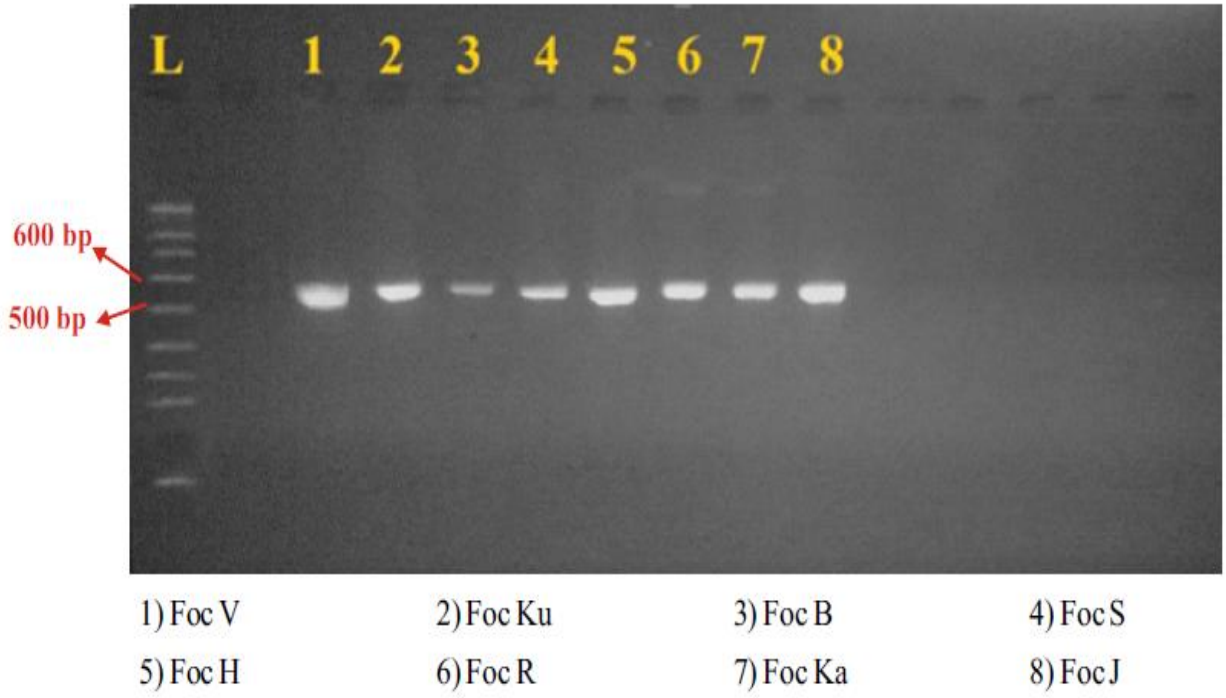
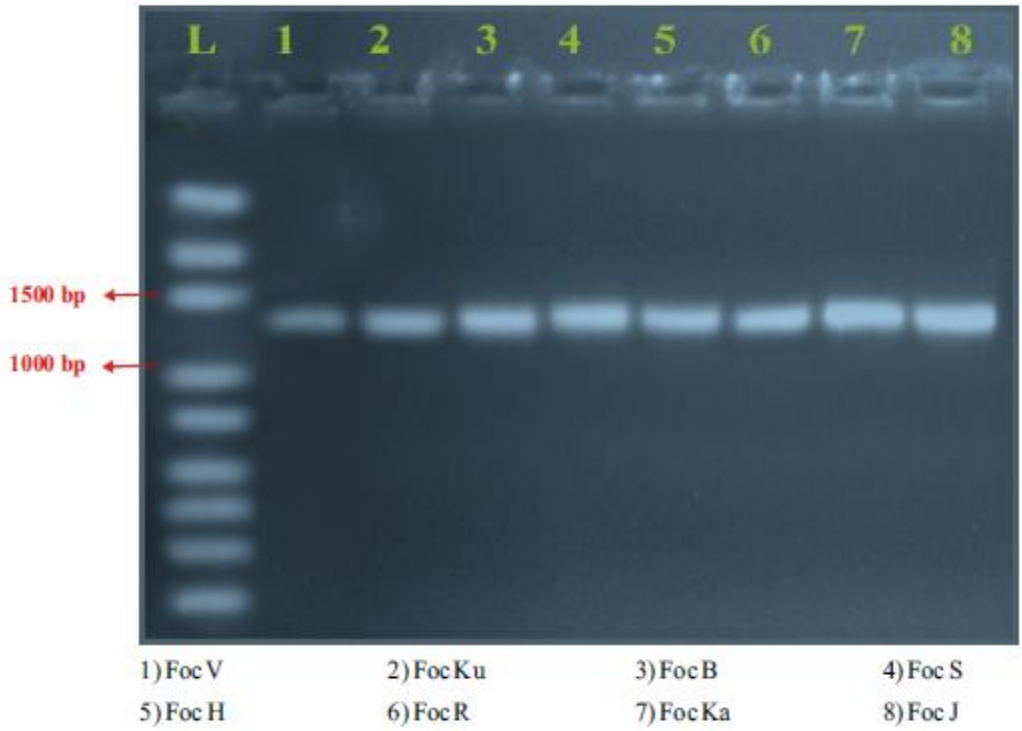


Plate.1b Specific amplification of *Fusarium oxysporum* f. sp. *ciceri* by species specific primers



The sequences of DNA of eight variants were compared using National Centre for Bioinformatics (NCBI) BLAST programme. Depending upon the sequence comparison, the identification of *Fusarium oxysporum* f. sp. *ciceri* was confirmed and all the ITS rDNA sequences of variants were confirmed as *Fusarium oxysporum* f. sp. *ciceri.*

The similarity coefficient among eight variants of *Fusarium oxysporum* f. sp. *ciceri* was up to 0.01. These variants were mainly categorized into cluster A and B. Cluster A contains six variants while cluster B contains two variants. The dendrogram constructed by UPGMA (Unweighted Pair Group Method Swith Arithmetic Mean) from the data it clearly shows that there are two clusters *viz.*, A and B (Fig. 1). Cluster A contained six variants *viz.*, Foc V, Foc Ku, Foc B, Foc S, Foc Ka and Foc H and cluster B contained two variants Foc R and Foc J.

From the DNA analysis and dendrogram it was evident that the eight variants were disparate in their genetic makeup which may be the reason for diversity among the variants. Further Foc V was clustered separately and had no genetic similarity with the any of the variants, thus making it as a distinct isolate leading to the possible existence of virulence among the variants in Northern Karnataka.

These results are well supported by the observations made by Naseema *et al.*, (2005), Honnareddy and Dubey (2006) and Thaware *et al.*, (2017).

References

Booth, C., 1971, The genus *Fusarium*, Commonwealth Mycological Institute, Kew (Surry), England, pp. 137.

Grewal, J. S. and Pal, M., 1970, Fungal diseases of gram and arhar. Proc. of 4th Workshop of Pulse Crops, Ludhiana, 169-170.

Honnareddy, N. and Dubey, S. C., 2006, Pathogenic and molecular characterization of Indian variants of *Fusarium oxysporum* f. sp. *ciceri* causing chickpea wilt. *Curr. Sci.*, 91(5): 85-89.

Jalali, B. L. and Chand, H., 1991, Chickpea wilt. In: Mukhopadhya, A. N., Chaube, H. S., Singh. U. S. and Kumar, J. (Eds.) *Diseases of International Importance*, Vol. 1, Prentice Hall, New Jersey, USA, pp. 7-10.

Naseema, A., Salim, A. M., Pradeep Krishnan, G. R. and Rajmohan, R., 2005, Molecular characterization of *Fusarium pallidoroseum*, a potent biocontrol agent of water hyacinth. *Pl. Dis. Res.*, 20(2): 210-212.

Nene, Y. L., Sheila, V. K. and Sharma, S. B., 1984, A world list of chickpea (*Cicer arietinum* L.) and pigeonpea (*Cajanus cajan* (L.) Millsp.) pathogens. ICRISAT Pulse Path. *Prog. Rep.*, 32: 19.

Nene, Y. L., Sheila, V. K. and Sharma, S. B., 1996, *A World List of Chickpea and Pigeonopea Pathogens*, 5th edn. ICRISAT, Patancheru, India, p. 27.

Patil, S., 2009, Effect of non-pathogenic *Fusarium* spp. in bio- control of *Fusarium* wilt of tomato. *M. Sc. (Agri.) Thesis*, Univ. Agric. Sci., Bangalore, Karnataka (India).

Singh, U., 1985, Nutritional quality of chickpea (*Cicer arietinum* L.): current status and future research needs. *Qualitas Plantarum Pl. Foods Human Nutri.*, 35: 339-351.

Thaware, D. S., Kohire, O. D. and Gholve, V. M., 2017, Cultural, morphological and molecular variability of *Fusarium oxysporum* f. sp. c*iceri* variants by RAPD Method. *Int. J. Curr. Microbiol. App. Sci.*, *6*(4): 2721-2734.

Trapero-Casas, A. and Jiménez-Díaz, R. M., 1985, Fungal wilt and root rot diseases of chickpea in southern Spain. *Phytopathol.*, 75: 1146–51.

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