Genetic resistance in chickpea (*Cicer arietinum* L.) against race 3 and 4 of Fusarium wilt

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Abstract

Chickpea (*Cicer arietinum* L.), a rabi season pulse crop in India is affected by Fusarium wilt that limits its productivity. In the present study, F_2 (BGD72 × WR315) and backcross (BGD72 × WR315) × BGD72 generations of chickpea were phenotyped against Fusarium wilt race 3 and race 4 isolates under sick plot and net house conditions. The plants segregated in 3:1 and 1:1 ratios in F_2 and BC_2F_1 generations for resistance and susceptibility to the isolates of both the races, indicating monogenic resistance to each race in these populations. The total 25 isolates were characterized morphologically on potato dextrose medium and by molecular means using internal transcribed spacer region (ITS) and beta-tubulin genes to confirm their species homology. The genetic analysis of F_2 and back cross populations for wilt resistance was carried out using 16 simple sequence repeats (SSR) primers, of which, 5 SSRs (TA194, TA22, TR19, TA110 and TA96) were found polymorphic (31%). The segregation ratio of individual markers using χ^2 test showed a monogenic inheritance in the F_2 and back cross population for resistance to both of the races. The three primers (TA22, TA194 and TA96) utilized against race-4 isolates showed specific amplification pattern in resistant genotypes only confirming that these markers are highly effective and may be used for large scale screening against Fusarium wilt resistance breeding and marker assisted selection in future breeding programs.

Introduction

Chickpea (*Cicer arietinum* L.), a cool season crop belonging to family *Fabaceae* and subfamily *Papilionaceae* is the second most widely cultivated pulse crop after common bean. India is the largest producer of chickpea where this crop has contributed to nearly 47% of the total pulse production in 2019-20 (Srivastava et al. 2021). Chickpea is rich in proteins, carbohydrates, dietary fibres, unsaturated fatty acids (linoleic and oleic acids), vitamins (vitamin A precursor β-carotene, riboflavin, niacin, thiamine and folate) and also some mineral nutrients (Ca, Mg, P and K). Chickpea is used as a feed for livestock and is very key pulse crop for improving soil fertility by the process of nitrogen fixation. Despite its broad adaptation, several biotic and abiotic stresses are responsible for hampering its production (Tarafdar et al. 2018; Caballo et al. 2019).

Among biotic stresses, Fusarium wilt (FW) caused by *Fusarium oxysporum* f. sp. *ciceri* (Foc) is the major disease limiting the chickpea productivity worldwide and causing annual yield losses ranging from 10 to 90% under favourable conditions depending upon disease severity (Sunkad et al. 2019). This disease can be found occurring during any stage of plant growth and the diseased plants can be seen in the form of field patchiness (Jimenez-Díaz et al. 2015). Disease severity and occurrence are based on inoculums load and varietal susceptibility. Large variability have been found in *F. oxysporum* in the form of eight races (0, 1 A, 1B/C, 2, 3, 4, 5 and 6) round the globe (Dubey et al. 2012; Rafiq et al. 2020) and two pathotypes,

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viz., wilting and yellowing (Jendoubi et al. 2017). *Foc* is a root inhabiting, seed borne pathogen which survives as dormant mycelium, in conidial forms and as chlamydospores (Patil et al. 2017). It has long term survival ability in the soil as there is no significant reduction in the disease incidence even after 3 years of crop rotation with sorghum, wheat and maize sometimes (Dubey et al. 2017).

The conventional method of chickpea breeding is time-consuming and suffers from the variability in pathogen races prevalent in different locations alongwith specific environmental factors that influence disease development (Soren et al. 2016). The deployment of wilt resistant varieties by the breeders is the most economic and sustainable strategy to mitigate the risk of this disease (Sharma et al. 2019; Jha et al. 2020), and depending upon different resistance sources, single or multiple genes have been found responsible for governing the resistance to FW (Singh et al. 2014).

Hence, molecular breeding approach is now becoming a feasible, rapid and promising strategy in engineering resistance varieties. Simple sequence repeats (SSRs) having tight linkage with *Fusarium* wilt resistance genes can be used for screening resistant genotypes in early stage of plant growth without subjecting them to pathogens. Such markers being effective, stable, simple and easy to operate, can be used to fasten the development of FW resistant and high yielding cultivars through marker assisted selection and gene pyramiding approach (Patil et al. 2014; Jingade and Ravikumar 2015). However, due to limited genetic polymorphism in the genome of cultivated chickpea species, the development of markers closely linked to wilt resistance is also limited (Li et al. 2015; Sahu et al. 2019). However comparison of different studies revealed that four FW resistance genes (*Foc1, Foc3, Foc4* and *Foc5*) cluster on the same linkage group (Millan et al. 2010; Varshney et al. 2014a; Garg et al. 2018)

indicating that wilt resistance could be a super loci functioning against multiple races.

Material and method

Plant material

Genetic material consisted of 140 plants in F_2 (BGD72 × WR315) and 44 plants in back cross (BC_2F_1) (BGD72 × WR315) × BGD72 generations grown during Rabi 2018-19 in the research area of Division of Genetics, ICAR-IARI, New Delhi. WR315 is a highly wilt resistant cultivar at 30 DAS and 60 DAS, whereas BGD72 is a moderately wilt resistant cultivar.

Pathogen characterization

A total of 25 isolates of *Fusarium oxysporum* f. sp. *ciceri* (Foc) representing race 3 and 4 collected from Central Zone and North Western Plains Zone of India, and maintained in the Pulse Pathology laboratory, Division of Plant Pathology, ICAR-IARI, New Delhi were used (Table 1). The isolates were characterized morphologically based on mycelia color, mycelia growth and the formation of microconidia, macroconidia and chlamydospores with the help of microscope under 40 × magnification. The molecular characterization of Foc isolates was undertaken by sequencing the internal transcribed spacer region (ITS) and beta-tubulin gene using the primers ITS-1 (5′-TCCGTAGGTGAACCTGCGG-3′), ITS-4 (5′-TCCTCCGCTTATTGATATGC-3′), Bt-1a (5′-TCCCCCGTCTCCACTTCTTCATG-3′) and Bt-1b (5′-GACGAGATCGTTCATGTTGAACTC-3′), respectively (Jiménez-Gasco et al. 2002). The genomic DNA from the Foc isolates was isolated from the air dried mycelial mat using

Table 1 Different isolates specific to Foc race 3 and race 4

Race 3	Race 4
Foc 1 (Jaipur, Rajasthan)	Foc 8 (IARI, New Delhi)
Foc 3 (Jaipur, Rajasthan)	Foc 9 (Hisar, Haryana)
Foc 5 (Alwar, Rajasthan)	Foc 29 (Ranchi, Jharkhand)
Foc 7 (Jaipur, Rajasthan)	Foc 30 (Faridkot, Punjab)
Foc 10 (Sikar, Rajasthan)	Foc 37 (Sikohpur, Haryana)
Foc 13 (Faridkot, Punjab)	Foc 41 (Hisar, Haryana)
Foc 14 (PAU, Ludhiana, Punjab)	Foc 53 (IARI Sickplot, New Delhi)
Foc 15 (KVK Faridkot, Punjab)	Foc 55 (IARI, New Delhi)
Foc 16 (Abohar, Punjab)	Foc 56 (IARI, New Delhi)
Foc 17 (Faridkot, Punjab)	Foc 103 (IARI Genetics Field, New Delhi)
Foc 20 (Faridkot, Punjab)	Foc 107 (IARI Genetics Field, New Delhi)
Foc 21 (Ferozpur, Punjab)	
Foc 42 (Sri Ganganagar, Rajasthan)	
Foc-154 (Jabalpur, Madhya Pradesh)	

modified CTAB method (Murray and Thompson 1980). The amplified products were sequenced using custom services of AgriGenome Labs, Pvt Ltd. Cochin, Kerala (India). The sequences thus obtained were subjected to BLASTn analysis tool of NCBI database and by using the UPGMA method of MEGAX software, a phylogentic tree was constructed.

Pathogenicity assay and disease assessment

The isolates were tested using artificially inoculated sick plot method to confirm their pathogenicity on JG-62, a susceptible chickpea cultivar. Control pots were maintained with only sterile soil. Seed germination, symptom development and finally wilt percentage disease index were recorded periodically up to the maturity of the crop. After detailed observation of symptoms produced by Foc, disease scoring was done on the main stem and each leaf separately 35 days after inoculation (DAI) using 0–5 scale given by Srinivasa et al. (2019).

Phenotypic and genotypic screening of F_2 and back cross generations

The parents, F_2 and back cross plants were evaluated in sick pot under net house conditions for disease reaction against Race 3 and 4 Foc isolates. The disease reaction was observed at 30th days of planting for early wilt symptoms as well as at 60th days of planting for late wilting symptoms. The scale value and PDI index used are in accordance with Srinivasa et al. (2019) for chi-square test analysis. For genotypic screening, 16 SSR markers tightly linked to the wilt resistance genes viz., *Foc1*, *Foc2*, *Foc3*, *Foc4* and *Foc5* (Sharma

and Muehlbauer 2007; Gowda et al. 2009) were selected (Table 2). DNA of parents (BGD 72 and WR 315), susceptible check (JG-62) and BC_2F_1 progeny was extracted by method of Simon and Muehlbauer (1997). The banding patterns obtained with different primers were scored in binary fashion and analyzed using NTSYS-PC software. Disease reaction as well as data generated from different markers was analyzed using chi-square test.

Results and discussion

Morphological characterization of Foc isolates

The 25 Foc isolates were fast growing and completely filled the plates 7 days after isolation when cultured on potato dextrose agar (PDA) media. The isolates were found highly variable in their morphological characteristics viz., macro and micro conidia sizes and mycelial growth characteristics in the photographs taken with microscope (Fig. 1). Macroconidia were thin walled, pointed at both the ends and measured 8–51 μm × 3–6 μm with 1 to 6 septa per conidium in an average while microconidia were oval to cylindrical, curved and measured 5.5–12 μm × 2-5.5 μm with no septation. Different isolates produced different types of growth like fluffy, compact, sparse and cottony. The mycelium was variable in pigmentations from white to yellowish white/ purple/ creamy/pink/violet. Dubey et al. (2010), Golakiya et al. (2018) and Venkataramanamma et al. (2019) also reported variation in morphological characters of colony of Foc isolates on the basis of type, growth pattern and pigmentation. Kaur et al. (2015) and Mahajan et al. (2019) assessed 24 and

Table 2 List of primers along with base sequences and their annealing temperatures

SSR marker	Forward primer sequence	Reverse primer sequence	Tm (°C)
TA96	TGTTTTGGAGAAGAGTGATTC	TGTGCATGCAAATTCTTACT	50
TA27	GATAAAATCATTATTGGGTGTCCTTT	TTCAAATAATCTTTCATCAGTCAAATG	51.5
TR59	AAAAGGAACCTCAAGTGACA	GAAAATGAGGGAGTGAGATG	51
TA110	ACACTATAGGTATAGGCATTTAGGCAA	TTCTTTATAAATATCAGACCGGAAAGA	53.5
H1B06	GACTCACTCTCCAAATGGAACC	AAGCCCATGAAAACCATATATTC	51.5
CaM1402	CACCCAAATCCCCAAA	TGCCTTTTGTATTTGAAAAATGTG	53
CaM1125	CACCCATTTTGATGGTCTGA	CAACAATTCCACTGCCTCTG	53.5
TA22	TCGTGTTTACTGAATGTGGA	TCTCCAACCCTTTAGATTGA	50.8
GA16	CACCTCGTACCATGGTTTCTG	TAAATTTCATCCTCTCCGGC	54
TAA60	TCATGCTTGTTGGTTAGCTAGAAC	GACATAATCGAGTTAAAGAAAA	54
TR19	TCAGTATCACGTGTAATTCGT	CATGAACATCAAGTTCTCCA	54
TA194	TTTTTGGCTTATTAGACTGACTT	TTGCCATAAAATACAAAATCC	49.7
TS82	TCAAGATTGATATTGATTAGATAAAAGC	CTTTATTTACCACTTGCACAACACTAA	52
H3A12	TCAATCTTTTGTTGTTACTATGAATCTG	AACCTTAGACTGTGTTCGCTGA	50.5
TA37	ACTTACATGAATTATCTTTCTTGGTCC	CGTATTCAAATAATCTTTCATCAGTCA	52.5
TS47	GTTAATATTTTTCCGCTTCGT	TCAAATTGTGTTAAAAATCAAAGTGTT	52

Fig. 1 **a** 14 isolates of Race 3 on PDA medium, **b** 11 isolates of Race 4 on PDA medium, **c** macroconidia (40X) of race 4, **d** microconidia (40X) of race 4, **e** Fungal mycelia (40X) of race 3, **f** chlamydospores (40X) of race 3
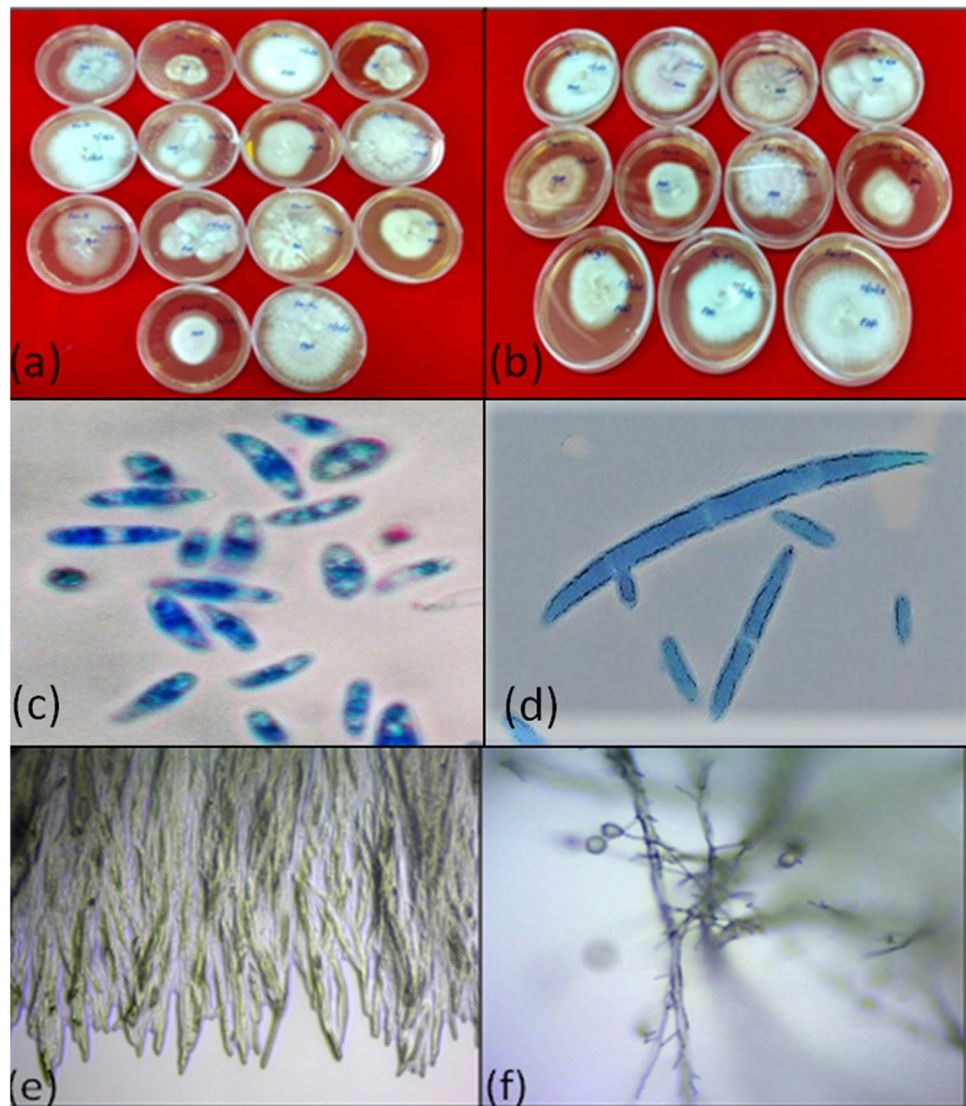
50 Foc isolates and reported sufficient variations for size of macroconidia, microconidia and chlamydospores. Certain differences in colony characters and septation in macroconidia in Foc isolates of race 1, 2 and 3 were observed by Singh et al. (2010).

Molecular characterization of Foc isolates

The internal transcribe spacer (ITS) region of rDNA as well as beta tubulin gene of Foc isolates were successfully amplified and sequenced (Fig. 2a, b). Based on ITS and beta tubulin gene sequences, out of 25 isolates, 14 representative isolates for ITS and 9 representative isolates for beta tubulin gene amplified product were sent for sequencing. The trimmed sequences subjected for molecular confirmation on NCBI data base using BLASTn tool showed a similarity index of more than 98–99% for ITS and more than 96% for beta tubulin gene and confirmed as *Fusarium oxysporum*

f. sp. *ciceris*. Kaur et al. (2015) assessed 24 isolates of *F. oxysporum* using their ITS genomic regions which were 99% similar to Foc sequences by BLAST analysis. Phylogenetic tree constructed using ITS and beta tubulin sequences (Fig. 3a, b) grouped the isolates into six and four clusters at 54% and 61% genetic similarity respectively which belonged to race 3 and race 4 sequences available from previous data base. Rakhonde et al. (2019) analysed 18 Foc isolates for their genetic diversity, which were then grouped into five clusters using UPGMA (unweighted paired group method) based on SSR markers. Younesi et al. (2021) selected 11 out of 36 Foc isolates for analysis of their β-tubulin gene regions and found that these 11 isolates were grouped into 5 different groups based on phylogenetic analysis. The isolates were scattered all across the trees and showed no correlation with the geographical distribution as well as physiological races, and clearly indicated the existence of more than one race in a specific geographical region. The present findings are in
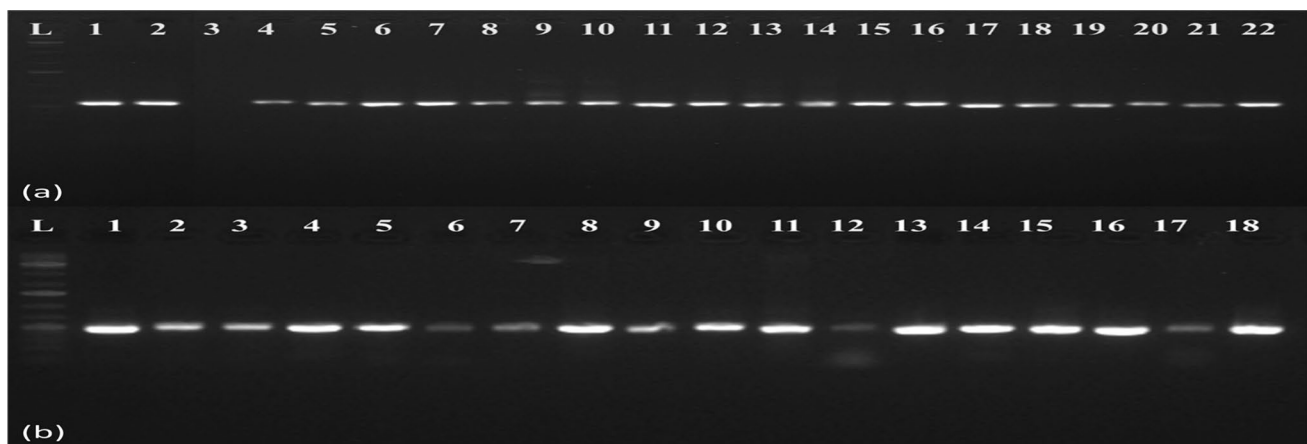
Fig. 2 Amplification of **a** ITS region and **b** beta tubulin gene of *Fusarium oxysporum* f. sp. *ciceri* isolates (lanes: L—100 bp ladder, 1-Foc-1, 2-Foc-3, 3-Foc-5, 4-Foc-7, 5-Foc-13, 6-Foc-14, 7-Foc-15, 8-Foc-16, 9-Foc-17, 10-Foc-20, 11-Foc-21, 12-Foc-39, 13-Foc-42, 14-Foc-154, 15-Foc-8, 16-Foc-9, 17-Foc-29 and 18-Foc-30, 19-Foc-37, 20-Foc-41, 21-Foc-53 and 22-Foc-55)

accordance with the observations made by earlier workers that molecular groups were not clearly correlated with the pathogenicity and geographic origin of the isolates (Cramer et al. 2003; Dubey et al. 2012; Alloosh et al. (2019).

Phenotypic evaluation of parents, F$_2$ and back cross generations for FW resistance

The phenotyping of parents, plants in F$_2$ and back cross generations for wilt reaction allowed a clear-cut classification of resistant and susceptible phenotypes. The number of plants showing wilt symptoms was recorded in percentage disease index on 30th day for early wilting and 60th day for late wilting. The range of wilt incidence varied from 0 to 100% for different isolates and PDI value of 10–25% were considered as resistant, whereas 25–100% as susceptible response. There were no wilting symptoms in WR 315 till maturity for both these races, BGD 72 showed less than 10% wilt incidence (1.28% and 7.73% at 30 and 60 DAS), whereas JG 62 had more than 88% wilting (89.23% and 97.67% at 30 and 60 DAS, respectively) and wilted completely in 25 days after sowing; reconfirming their wilt resistance and susceptibility, respectively. Against race 3 isolates, 38 plants in F$_2$ generation while 19 plants in BC$_2$F$_1$ generation showed resistance while rest of the plants showed susceptible response (Table 3). Similarly, 43 plants in F$_2$ generation and 21 plants in BC$_2$F$_1$ generation showed resistance against race 4 isolates while rest of the plants showed susceptible response in both the generations. The plants in F$_2$ generation segregated in 3:1 ratio for resistance and susceptibility whereas plants in BC$_2$F$_1$ generation segregated in 1:1 ratio to Foc 3 and 4 isolates, indicating that monogenic resistance was there in these populations against each race. Gowda et al. (2009) reported the resistance to be monogenic for resistance and

susceptibility as seen from segregation of RILs in 1:1 ratio from their study. In both the generations, the disease reaction data indicated a good fit to the 1:1 segregation ratio by chi-square analysis, which is expected for single genes conferring resistance.

Molecular marker (SSR) analysis

Out of 16 SSR primers used, 13 SSRs (81%) produced scorable bands in parental genotypes, of which 5 SSRs (TA194, TA22, TR19, TA110 and TA96) were polymorphic (31%) and the remaining 8 gave monomorphic bands. To detect the association and segregation pattern of DNA markers with FW resistance for marker genotyping, these polymorphic markers were used on the back cross population. The PCR amplification of TA22 primer generated two alleles, out of which the allele of 250 bp was observed only in resistant genotypes (Fig. 4). The amplification sizes of primers, TR19, TA194 and TA96 as characterized in *ciceri* sp. were 234, 280 and 265 bp, respectively, which are closely consistent with reported results of Padaliya et al. (2013). These three primers showed specific amplification pattern in resistant genotypes only, which can be effectively utilized for large scale screening in disease resistance breeding programme of chickpea. Lal et al. (2021) while studying RILs of chickpea found 12 markers linked to wilt resistance in chickpea, out of which, TA96, CS27, TA110 and TA59 were found more consistently related. DNA marker based tagging of resistance genes for Foc races 1–5 has been established using various SSR markers in recombinant inbred lines (RILS) populations generated from various resistant and susceptible parental combinations of chickpea (Sharma et al. 2004; Iruela et al. 2007; Gowda et al. 2009).
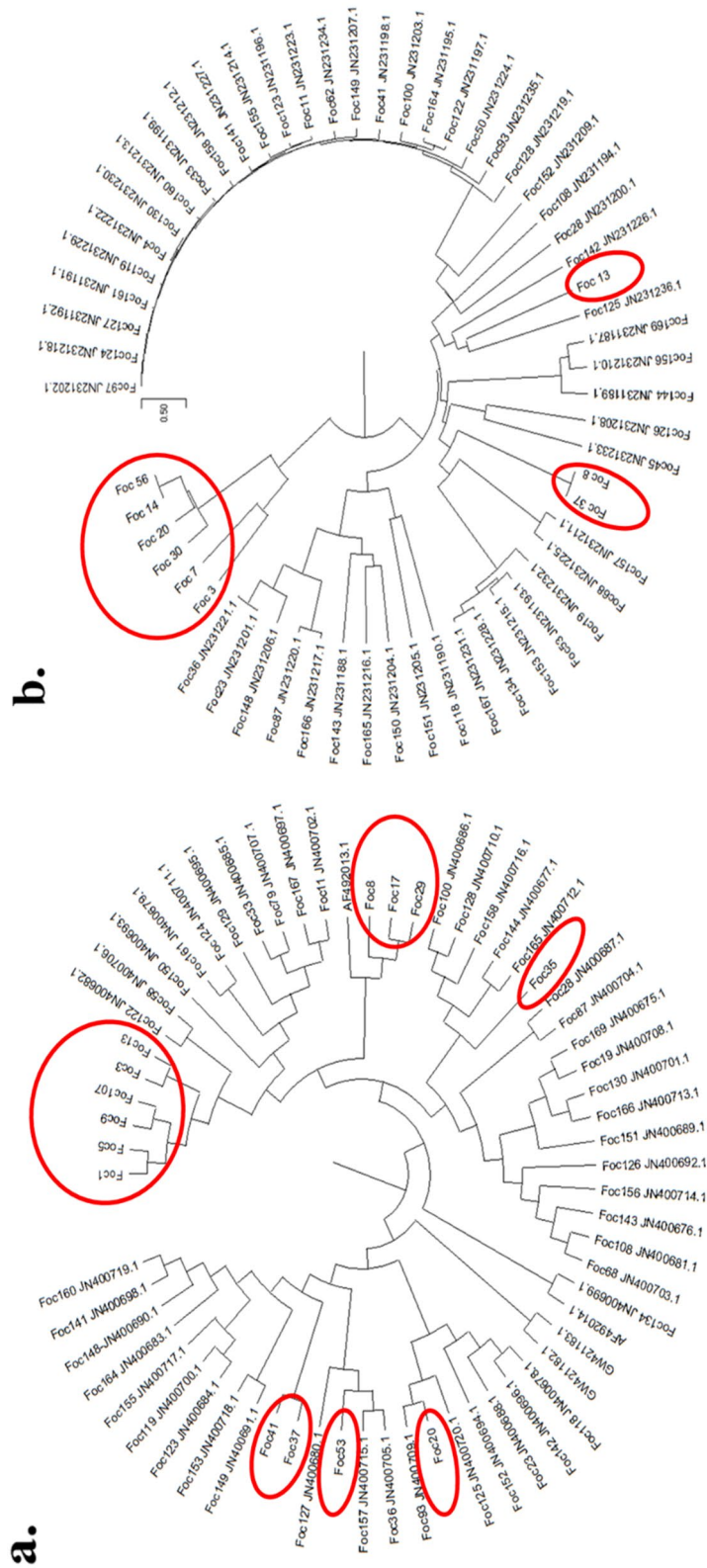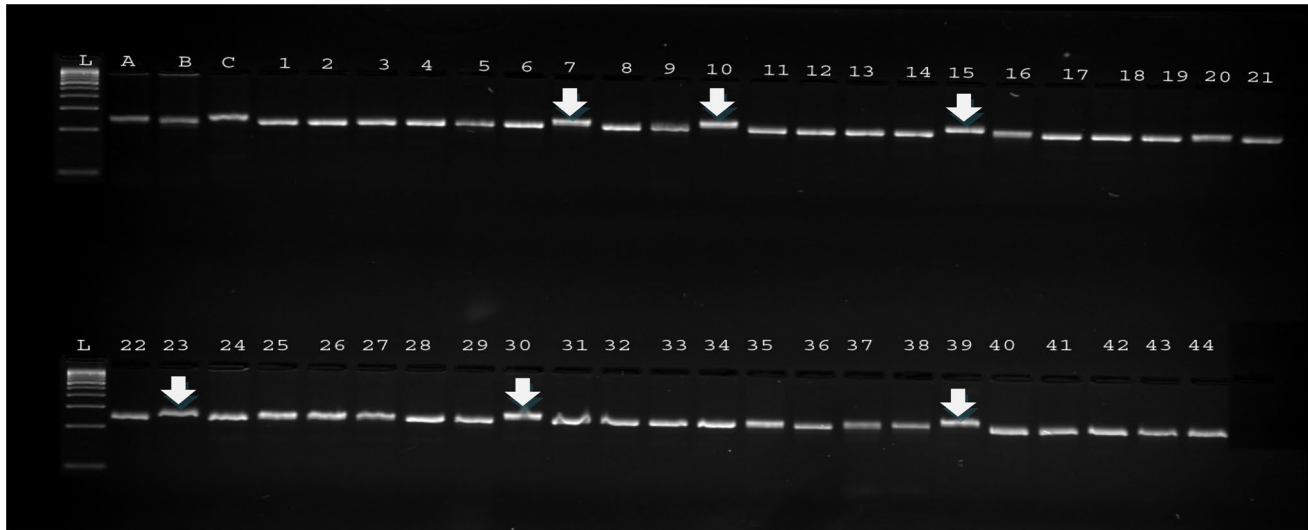
Fig. 3 Phylogentic tree constructed based on **a** ITS region and **b** beta tubulin gene sequences of *F. oxysporum* f. sp. *ciceri* isolates through MEGAX software using UPGMA method

Table 3 Phenotypic segregation of F_2 and BC_2F_1 populations for resistance to race 3 and 4 of fusarium wilt

Race	Population	No. of resistant plants (10–25% PDI)	No. of susceptible plants (25–100% PDI)	Total no. of plants	χ^2 value
3	F_2	38	102	140	0.34
	BC_2F_1	19	25	44	0.82
4	F_2	43	97	140	2.44
	BC_2F_1	21	23	44	0.09

Fig. 4 Agarose gel electrophoresis result showing segregation pattern among **a** JG62, **b** BGD72, **c** WR315 and 44 BC_2F_1 progenies derived from cross (BGD72 × WR315) × BGD72 for primer TA22

A monogenic inheritance was observed in the back cross population for resistance against these two Foc races when the segregation ratio of individual marker was tested using χ^2 test. Sharma et al. (2005) observed monogenic i.e., 1:1 ratio for single gene resistance to Foc race 3 (Foc3) and race 4 (Foc4), whereas Barman et al. (2014) reported monogenic ratio to Foc race 1. The results showed that these markers were highly efficient for the prediction of desired genotypes, therefore, these can be used for marker assisted selection (MAS). In the present study, the percent polymorphism obtained was very low (31%), except for markers associated with resistance genes, which might be due to the involvement of two desi cultivars in the development of the back cross population differing for wilt reaction only. In earlier reports of Radhika et al. (2007) (9.5% and 11.57%), Gowda et al. (2009) (13.45%) and Nayak et al. (2010) (16.7%) also, such a very low level of polymorphism was reported.

Conclusions

The fusarium wilt appears to be an important disease that calls for better attention in terms of economical management with use of resistant varieties for the effective management of the disease and to stabilize chickpea yields. In the present study, the race 3 and 4 Foc isolates were characterized at both the morphological and molecular levels because only morphological characterization of isolates is not enough to define their identity, therefore, amplification and sequencing of DNA fragments containing the ribosomal ITS and beta-tubulin at molecular levels is necessary to prove their species homology. Use of molecular tools offers great potential for chickpea improvement, specifically by identifying molecular markers closely linked to genes controlling fusarium wilt. Three molecular markers

(TA-22, TA-194 and TA-96) showed specific amplification pattern in resistant genotypes only, which can be effectively utilized for large scale screening in disease resistance breeding as well as for marker assisted breeding programme of chickpea. However, a higher density of closely-linked markers in the area of these genes is still necessary to detect polymorphisms for marker-assisted selection (MAS) in different genetic backgrounds.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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