

Original Research Article

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Marker Trait Correlation Study for *Fusarium* wilt Resistance in Chickpea (*Cicer arietinum*)

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ABSTRACT

The investigation was focused on transfer of the *Fusarium* wilt resistance into elite cultivar. Screening of chickpea parents (ICC 506 EB and Vijay), 196 RIL's (Obtained from ICRISSAT, Hyderabad), F₂ and BC₁F₁ populations for *Fusarium* wilt resistance were done by Pot culture method and wilt sick plot method. The BC₁F₁ segregated in 1:1 ratio for resistance and susceptibility and F₂ progenies segregated in a ratio of 1 resistant and 3 susceptible. The RILs closely fit a 1:1 segregation ratio for resistance and susceptibility indicating that resistance to *Fusarium* wilt was monogenic with the recessive allele conferring resistance to *Fusarium* wilt in this population. The parents were screened with 43 SSR primers. 22 markers were identified polymorphic. The polymorphism ranged from 57.14 to 100.00 per cent. The PIC scores of SSR markers ranged between 0.0371 and 0.9226. The BC₁F₁ population screened with three polymorphic foreground markers (TR19, TA110 and GA16) and four polymorphic background markers (TS82, TA194, TA135 and TA 22). The reported markers linked to susceptibility and resistance proved their effectiveness and further can be exploited for maker assisted selection (MAS) of *Fusarium* wilt resistance breeding in chickpea.

Keywords

Fusarium wilt,
SSR, RIL's, F₂,
BC₁F₁, MAS, PIC

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Introduction

Chickpea (*Cicer arietinum*) is third most important grain legume crop grown in the arid and semi-arid regions of the world. It is one of the important grain legume crops of India which plays an important role in food security and balanced diet.

Chickpea holds prestigious position among all legume crops because it plays an important role in food security and balanced diet. It is virtually an indispensable item in the kitchen

and is considered as "king of pulses" (Bhatt and Patel, 2001). Two main types of chickpea cultivars are grown globally- kabuli and desi, representing two diverse gene pools (Pundir *et al.*, 1985). It serves as an important source of protein in human diet and plays an important role in the enrichment of soil fertility. Chickpea seeds containing 20–30% protein, about 40% carbohydrates, 3–6% oil, 6% crude fiber and 3% ash (Gil *et al.*, 1996).

Among the biotic stresses that affect chickpea (*Cicer arietinum*), *Fusarium* wilt (*Fusarium*

oxysporum) is a major yield-limiting factor. *Fusarium* wilt is a soilborne disease that causes severe yield losses. The pathogen is both seed and soil borne, survives in the soil for more than six years in the absence of susceptible host plants (Haware *et al.*, 1986).

Eight physiological races of the pathogen (race 0, 1A, 1B/C, 2, 3, 4, 5 and 6) have been identified by reaction on set of differential chickpea cultivars (Jimenez-Diaz *et al.*, 1989). Races 0 and 1B/C induce yellowing symptoms, whereas remaining races inducing wilting.

The use of DNA-based markers for the genetic analysis and manipulation of important agronomic traits has become an increasingly useful tool in plant breeding. Marker-assisted selection (MAS) is a new paradigm in plant breeding. Although chickpea improvement for *Fusarium* wilt resistance through conventional breeding and hybrid technology is ongoing, molecular breeding should accelerate utilization of the substantial variability among the chickpea landraces and germplasm lines. The application of biotechnology would be a better choice to minimize the incidence of disease and pest in agricultural crops. The use of molecular markers in crop cultivars gives an additional advantage in characterizing, selection and maintaining the genetic purity.

Materials and Methods

Experimental material

The experimental chickpea seed material for the present investigation comprised of a mapping population in the form of 196 recombinant inbred lines (RILs) derived from a cross between Vijay (resistant to *Fusarium* wilt) X ICC 506 EB (susceptible to *Fusarium* wilt). The experimental material was kindly provided by Dr. H. C. Sharma, Principle Scientist, Entomology from ICRISAT.

Collection of diseased samples

Chickpea wilt infected samples were collected from the field of Pulses Research Unit, Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola, Maharashtra. The samples were collected during chickpea growing season in the year 2012-2013.

Preparation of Mass inoculums

Purified cultures of six isolates of *Fusarium oxysporum* f. sp. *ciceri* were mass multiplied separately on sorghum sand medium (1 part partially broken sorghum grain + 3 part sand + distilled water to moisten the media). The media was prepared by mixing broken sorghum grains with clean sand in plastic tub followed by moistening with distilled water. About 500 g mixture was transferred in 2000 ml Erlenmayer flask plugged using non absorbent cotton and sterilized in an autoclave at 15 p.s.i. for 30 minutes consequently for two days.

It was allowed to cool and the flasks containing the sterilized media were inoculated with mycelial disc of pure culture of *F. oxysporum* f. sp. *ciceri* (5 mm diameter) and incubated at $27 \pm 2^{\circ}\text{C}$ for 15 days. Sufficient quantity of inoculum was prepared and used for preparing sick pots required in pot experiments.

Preparation of sick soil

Soil was collected in gunny bags and sterilized in autoclave at 1.05 kg/cm^2 for one hour consequently for three days. Sand was added to the soil to facilitate proper drainage and aeration in pots.

Finally the mass multiplied fungus inoculum was added in 1: 10 proportion to soil and thoroughly mixed, thus the soil was made sick.

Pathogenicity test

Plastic pots of size 10 cm diameter were taken and surface sterilized with 0.1% HgCl₂. The sick soil was filled in sterilized pots 1/4th of its capacity. The pots were given water lightly and incubated for 4 days. Five seeds of susceptible chickpea cultivar JG-62 were surface disinfected with 4% sodium hypochlorite solution for 30 seconds and sown in pots each isolate in 3 replications. The seedlings maintained in sterilized soil without inoculums were served as control. Plants were observed periodically upto 30 days after sowing (DAS) for wilt symptoms and disease incidence (%) and total mortality was calculated. Different isolates of *F. oxysporum* were tested by sick soil method for their virulence on susceptible variety JG-62. The percent wilting was recorded on the basis of healthy and wilted plants. Wilt incidence was calculated by using formula,

The isolates of *Fusarium oxysporum* f. sp. *ciceri* were tentatively divided into three groups on the basis of virulence as Non pathogenic isolates (0-10 percent), Moderately pathogenic isolates (10.1-30 percent), Highly pathogenic isolates (>30 percent)

Screening of chickpea parents and RIL's by Pot culture method

Screening of chickpea parents (ICC-506 and Vijay) and 196 RIL's (Obtained from ICRISAT, Hyderabad) for wilt resistance were done by Pot culture method in green house. Plastic pots of size 10 cm diameter were taken and surface sterilized with 0.1% HgCl₂. The sick soil was filled in sterilized pots upto 1/4th of its capacity. The pots were watered lightly and incubated for 4 days. Chickpea seeds of parents (ICC 506 and Vijay) and 196 RIL's of susceptible chickpea cultivar JG-62 were surface disinfected with 4% sodium hypochlorite solution for 30 seconds and sown

in pots in 3 replications (10 seeds per pot). The seedlings maintained in sterilized soil without inoculums were served as control. Plants were observed periodically upto 30 days after sowing (DAS) for wilt symptoms and disease incidence (percent) and total mortality were calculated. Reactions were graded as resistant (0-10 percent wilt), moderately resistant (10.1 to 30 percent wilt) and susceptible (> 30 percent wilt) (Anonymous, 2016).

Screening of chickpea genotypes in Field

Chickpea parents (ICC 506 and Vijay) and 196 RIL's (Obtained from ICRISAT, Hyderabad) were screened in wilt sick plot condition at Pulses Research Unit, Dr. PDKV, Akola. A field screening technique for wilt screening developed at ICRISAT was adopted in the present studies (Nene *et al.*, 1980). In this screening technique a wilt susceptible check (JG-62) was sown intermittently after every five test entries so as to monitor the disease pressure. Sowing of chickpea germplasm was completed in November, 2012 with two replications of row length 3 m at 30x10 cm spacing. The seed emergence was recorded 18 days after sowing. Observation on number of plants wilted was recorded at 30 days and 60 days after sowing. The percent wilt incidence was calculated on the basis of initial plant count and total number of wilted plants in each genotype and graded as follows (Anonymous, 2016).

Crossing of selected genotypes

Crossing chickpea is tedious and time consuming and a crossed pod generally produces only one seed. Emasculation is required for artificial hybridization in chickpea. The crossing programme was carried out at experimental field, Pulses Research Unit, Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola during rabi 2012-

2013 to 2014-2015. The crosses were made during rabi 2012-2013, to obtain first filial (F_1) generation. The F_1 was grown to produce F_2 population. The F_1 's were crossed with above female to produce BC_1F_1 backcross populations during rabi 2013-2014. All these populations viz., P_1 , P_2 , F_1 , BC_1F_1 were sown in rabi 2014-2015.

Parental polymorphism study

The parents of the mapping population ICC 506 EB and Vijay were screened with 43 SSR markers for identification of the polymorphic markers.

Polymorphism study of RIL's using polymorphic markers

The mapping population derived from ICC 506 EB and Vijay was screened with 22 SSR (Table 1) markers which were found polymorphic between parents. The data generated from polymorphism of RIL's was used for further analysis.

Methodology for SSR Markers

For SSR marker studies, genomic DNA was isolated from each of the parent and 196 RILs using a modified CTAB method (Sharma *et al.*, 2002). Forty three SSR primer pairs were used for the present investigation. The sequence information for these primers was obtained from reviewed literature, while the synthesis was done from Genaxy Scientific Pvt. Ltd., India.

Scoring of SSR amplified bands and genotyping

The polymorphic SSR markers identified to be polymorphic after parental polymorphism analysis were utilized further for the molecular data scoring to know the genotyping of the 196 RILs based on morphological data and the

parents. The gel image of SSR analysis were captured and visualized under light in gel documentation system (Biorad). Data was scored as the presence (1) or absence (0) of individual band for each isolate. The similarity index was calculated and the data was used to generate similarity coefficient using simple matching coefficient based on SSR bands scoring. The similarity coefficient between each pair of accessions were then used to construct a dendrogram using the Unweighted Pair Group Method with Arithmetic Average (UPGMA)

Results and Discussion

Chickpea wilt infected samples were collected from the field of Pulses Research Unit, Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola, Maharashtra during chickpea growing season in the year 2012-2013. The tissue isolation method was used for isolation of *Fusarium oxysporum* f.sp. *ciceri* from infected plants showing typical wilt symptoms. The pure culture thus obtained was identified as *Fusarium oxysporum* f.sp. *ciceri* on the basis of morphological characters reported by Booth (1977) (Plate 1).

The Pathogenicity test of isolates of *F. oxysporum* f.sp. *ciceri* isolated was tested by using susceptible cultivar JG-62. The samples of *Fusarium oxysporum* f. sp. *ciceri*, proved to be pathogenic to susceptible cultivar JG-62 (64.28%). The isolate from Pulses Research Unit, Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola, Maharashtra were used for further screening of parents and RIL population.

Screening of chickpea parents (ICC 506 EB and Vijay) and 196 RIL's (Obtained from ICRISAT, Hyderabad) for wilt resistance were done by Pot culture method (Fig. 1) in green house as well as in wilt sick plot condition (Fig. 2) at Pulses Research Unit, Dr. PDKV,

Akola. A field screening technique for wilt screening developed at ICRISAT was adopted in the present studies (Nene *et al.*, 1991). JG-62 a highly susceptible genotype was used as a check.

Among the 196 RILs, 22 RILs were resistant, 55 RILs were moderately resistant and 119 were susceptible. The RILs also segregated in 1:1 ratio for resistance and susceptibility, indicating that resistance to *Fusarium* wilt was monogenic in this population. The details of the experiment are given in Table 2 (Plate 2, 3 and 4).

The crossing programme was carried out at experimental field, Pulses Research Unit, Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola. Total 310 flowers were pollinated to obtain F₁ and 260 flowers were pollinated to obtain BC₁F₁. Percent pod set observed for F₁ was 20.64% and for BC₁F₁, % pod set was 18.64% (Table 3).

Screening of parents (Vijay and ICC 506 EB), BC₁F₁ and F₂ generations for wilt resistance were done by Pot culture method in green house. JG-62 a highly susceptible genotype was used as a check. Among the 51 BC₁F₁ 26 plants were resistant and 25 were susceptible. The susceptible parent ICC 506 EB, showed 83.33 percent wilting in 30 days after sowing, whereas Vijay was resistant till maturity. Among the 136 F₂, 107 were found resistant and 29 were susceptible.

The BC₁F₁ segregated in 1:1 ratio for resistance and susceptibility and F₂ progenies segregated in a ratio of 3 susceptible and 1 resistant. The RILs also closely fit a 1:1 segregation ratio for resistance and susceptibility indicating that resistance to *Fusarium* wilt was monogenic in this population. The data revealed segregation of a single gene with the recessive allele conferring resistance to *Fusarium* wilt (Table 4).

SSR markers were found to be useful genetic markers as revealed by their co dominance, high frequency, and high polymorphism. The parents were screened with 43 SSR primers to identify the polymorphic markers associated with *Fusarium* wilt resistance component traits. Out of 43 SSR markers screened, 22 polymorphic markers were identified. Genetic variation was detected among 196 RIL's using identified polymorphic SSR marker for *Fusarium oxysporum* f.sp. *ciceri*. The segregation of the 22 polymorphic markers across the mapping population (RIL) was analyzed using the PCR. The polymorphic markers were separated on 8 percent denaturing PAGE (Poly acrylamide gel electrophoresis).

All primers showed good polymorphism and produced scorable bands with high degree of polymorphism. Twenty two SSR primer pairs produced total of 92 alleles across 196 RIL's, of which 81 were found polymorphic. Maximum of 8 alleles were amplified by primer pairs of TA 200 and the least alleles 2 were amplified by CaSTMS21, TA8, CaSTMS2, CaSTMS15, TA135 and TA71 primer. Total number of alleles generated per primer pair ranged from 2 to 8, with an average of 4.18 alleles per primer (Table 5). Twenty two SSR primer pairs produced total of 92 alleles across 196 RIL's, of which 81 were found polymorphic. The extent of polymorphism ranged from 57.14 per cent (TA103) to 100.00 per cent with an average of 91.97 per cent. The size of amplified alleles ranged between 114-300 bp. Genetic diversity for a specific marker was evaluated by PIC. The range of PIC scores of SSR markers ranged between 0.0371 (TR19) to 0.9226 (TR1). The average PIC value of primers was observed to be 0.2294. Results of percent polymorphism by SSR marker earlier reported by 100 percent by Singh *et al.*, (2008), 93 percent by Datta *et al.*, (2010), 83 percent by Rizvi *et al.*, (2014).

Table.1 List SSR primers revealed polymorphism among parents

Sr. No.	Primer Name	Forward and Reverse sequence
1	TR19	F- TCAGTATCACGTGTAATTCGT R- CATGAACATCAAGTTCTCCA
2	TA 96	F- TGTTTTGGAGAAGAGTGATTC R- TGTGCATGCAAATTCTTACT
3	TA 27	F- GATAAAATCATTATTGGGTGTCCTTT R- TTCAAATAATCTTTCATCAGTCAAATG
4	TA 59	F- ATCTAAAGAGAAATCAAATTGTGCGAA R- GCAAATGTGAAGCATGTATAGATAAAG
5	TS 82	F- TCAAGATTGATATTGATTAGATAAAAAGC R- CTTTATTTACCACTTGCACAACACTAA
6	TA 194	F- TTTTTGGCTTATTAGACTGACTT R- TTGCCATAAAAATACAAAATCC
7	TA 110	F- ACACTATAGGTATAGGCATTTAGGCAA R- TTCTTTATAAATATCAGACCGGAAAGA
8	TA 103	F- TGAAATATCTAATGTTGCAATTAGGAC R- TATGGATCACATCAAAGAAATAAAAT
9	TA 200	F- TTTCTCCTCTACTATTATGATCACCAG R- TTGAGAGGGTTAGAACTCATTATGTTT
10	GA 16	F- CACCTCGTACCATGGTTTCTG R- TAAATTTTCATCCTCTCCGGC
11	TA 37	F- ACTTACATGAATTATCTTTCTTGGTCC R- CGTATTCAAATAATCTTTCATCAGTCA
12	TA72	F- GAAAGATTTAAAAGATTTTCCACGTTA R-TTAGAAGCATATTGTTGGGATAAGAGT
13	TA130	F- TCTTTCTTTGCTTCCAATGT R-GTAAATCCCACGAGAAATCAA
14	TA71	F- CGATTTAACACAAAACACAAA R-CCTATCCATTGTCATCTCGT
15	TA22	F- TCTCCAACCCTTTAGATTGA R-TCGTGTTTACTGAATGTGGA
16	TA135	F- TGGTTGGAAATTGATGTTTT R-GTGGTGTGAGCATAATTCAA
17	TR1	F- CGTATGATTTTGCCGTCTAT R-ACCTCAAGTTCTCCGAAGT
18	CaSTMS2	F- ATTTTACTTTACTACTTTTTTCCTTTC R-AATAAATGGAGTGAAATTTTCATGTA
19	CaSTMS15	F- CTTGTGAATTCATATTTACTTATAGAT R-ATCCGTAATTTAAGGTAGGTTAAAATA
20	CaSTMS21	F- CTACAGTCTTTTGTCTTCTAGCTT R-ATATTTTTTAAGAGGCTTTTGGTAG
21	TA8	F- AAAATTTGCACCCACAAAATATG R- CTGAAAATTATGGCAGGGAAAC
22	TA21	F- GTACCTCGAAGATGTAGCCGATA R- TTTTCCATTTAGAGTAGGATCTTCTTG

Table.2 Screening of Parents and RIL populations in wilt sick pot

	% Mean wilt incidence
Mean of RIL's	38.60 (37.93)*
Vijay	10 (18.43)
ICC 506 EB	83.33 (66.14)
JG-62 (check)	90 (90.00)
SE ±	3.62
C. D. @ 5%	10.08

* - transformed values

Table.3 Observations for cross ICC-506 × Vijay

Crosses	ICC 506 EB × Vijay (F ₁)	F ₁ × ICC 506 EB (BC ₁ F ₁)
No. of Pollinations	310	260
No. of Pod set	64	48
No. of seed set	69	52
% Pod set	20.64	18.64

Table.4 Inheritance of wilt resistance in a cross ICC-506 x Vijay

	Total plants	Wilted plants	Non wilted plants	Expected ratio	df	x ²	P-value
RIL's	196	119	77	1:1	1	9.433	0.0021
F ₂	136	107	29	3:1	1	0.1985	0.655
BC ₁ F ₁ (F ₁ × ICC 506 EB)	51	25	26	1:1	1	0.078	0.780

df= 1; P=0.05; x²=3.841

Table.5 Percentage polymorphism of different SSR primers

Sr. No.	Primers	Total no. of amplicons	Polymorphic alleles	Percentage of polymorphism (%)	PIC
1.	TR19	5	5	100.00	0.1883
2.	TA 96	5	4	80.00	0.0994
3.	TA 27	6	4	66.66	0.0371
4	TA 59	7	5	71.42	0.0713
5.	TS 82	4	4	100.00	0.1775
6.	TA 194	7	7	100.00	0.0702
7.	TA 110	3	3	100.00	0.2332
8.	TA 103	7	4	57.14	0.0681
9.	GA 16	3	3	100.00	0.2769
10.	TA 200	8	7	87.50	0.0601
11.	TA 37	5	5	100.00	0.1911
12.	TA 72	7	6	85.71	0.0687
13.	TA130	2	2	100.00	0.2385
14.	TA 71	2	2	100.00	0.4872
15.	TA 22	3	3	100.00	0.2704
16.	CaSTMS15	2	2	100.00	0.4864
17.	TA135	2	2	100.00	0.4875
18.	TR1	4	3	75.00	0.9226
19.	CaSTMS2	2	2	100.00	0.4069
20.	TA21	4	4	100.00	0.1929
21.	CaSTMS21	2	2	100.00	0.4001
22.	TA8	2	2	100.00	0.3421
	Total	92	81	-	-
	Average	4.18	3.68	91.97	0.2294

Table.6 Foreground selection for ICC 506 EB X Vijay derived BC₁F₁ progenies

Sr. No.	Particulars	ICC 506 EB X Vijay
1	Number of plants screened	51
2	Number of polymorphic marker used	3
3	Scorable marker data points generated	149
4	Number of progeny satisfying the foreground selection for all the targeted QTL regions	8
5	Marker status of selected plants at target QTL regions	Heterozygous

Table.7 Background selection for ICC 506 EB X Vijay derived BC₁F₁ progenies

Sr. No.	Particulars	ICC 506 EB X Vijay
1	Number of plants screened	51
2	Number of polymorphic marker used	4
3	Scorable marker data points generated	198
4	Number of progeny satisfying the foreground selection for all the targeted QTL regions	3
5	Marker status of selected plants at target QTL regions	Heterozygous

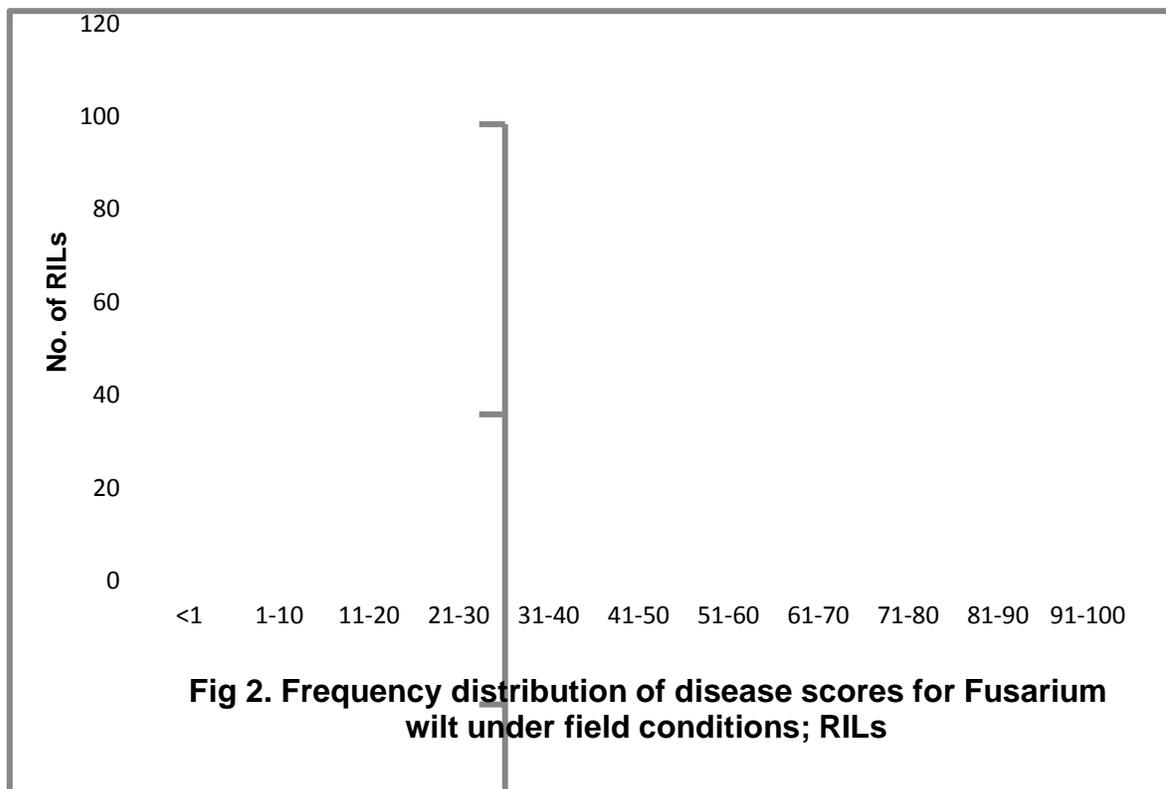
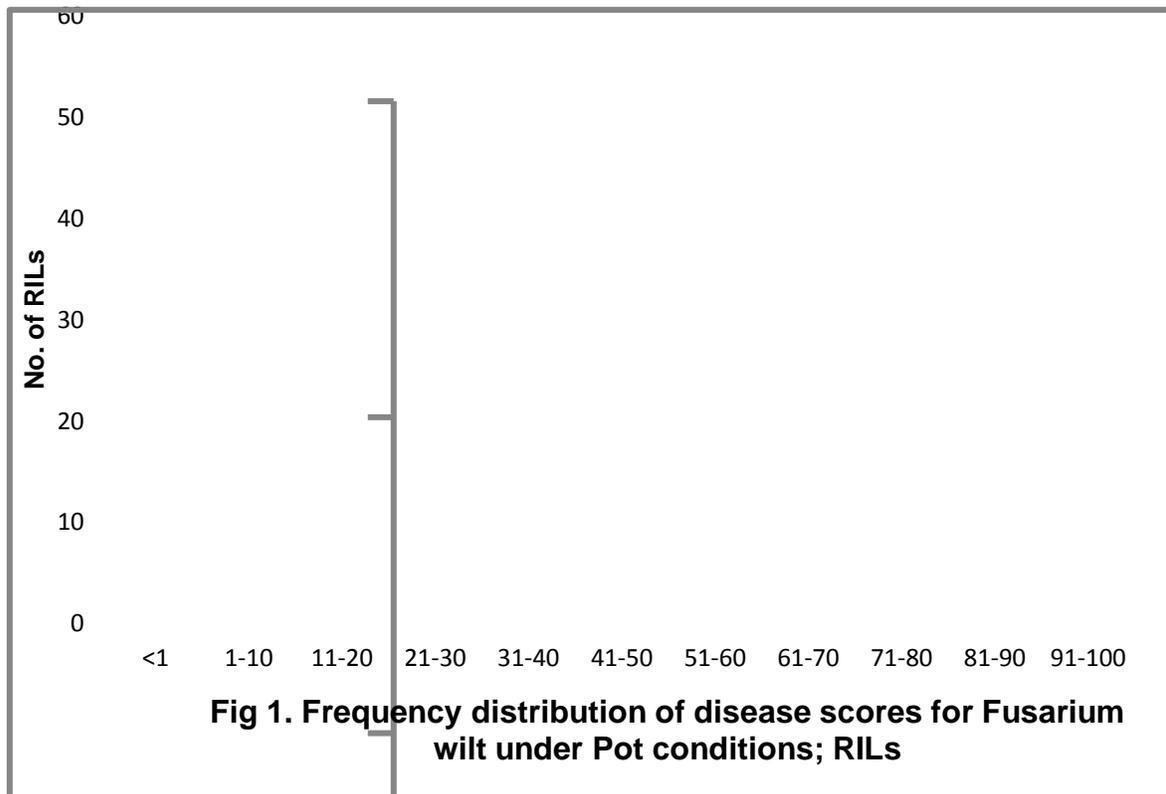


Fig.3 Dendrogram constructed using Jaccard's similarity coefficient and UPGMA clustering for 196 RIL's based on SSR analysis

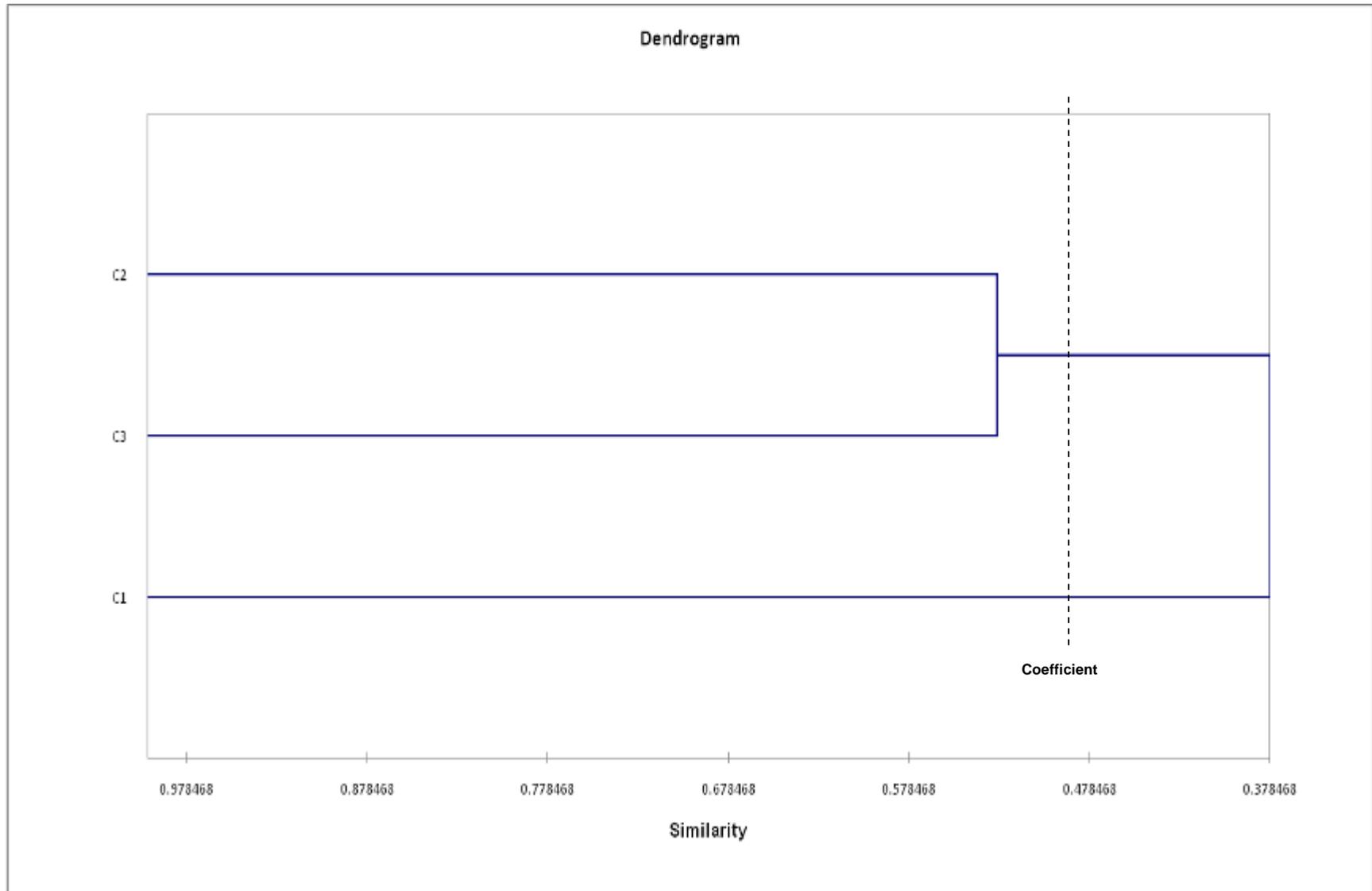


Fig.4 Dendrogram constructed using Jaccard's similarity coefficient and UPGMA clustering for 196 RIL's based on SSR analysis

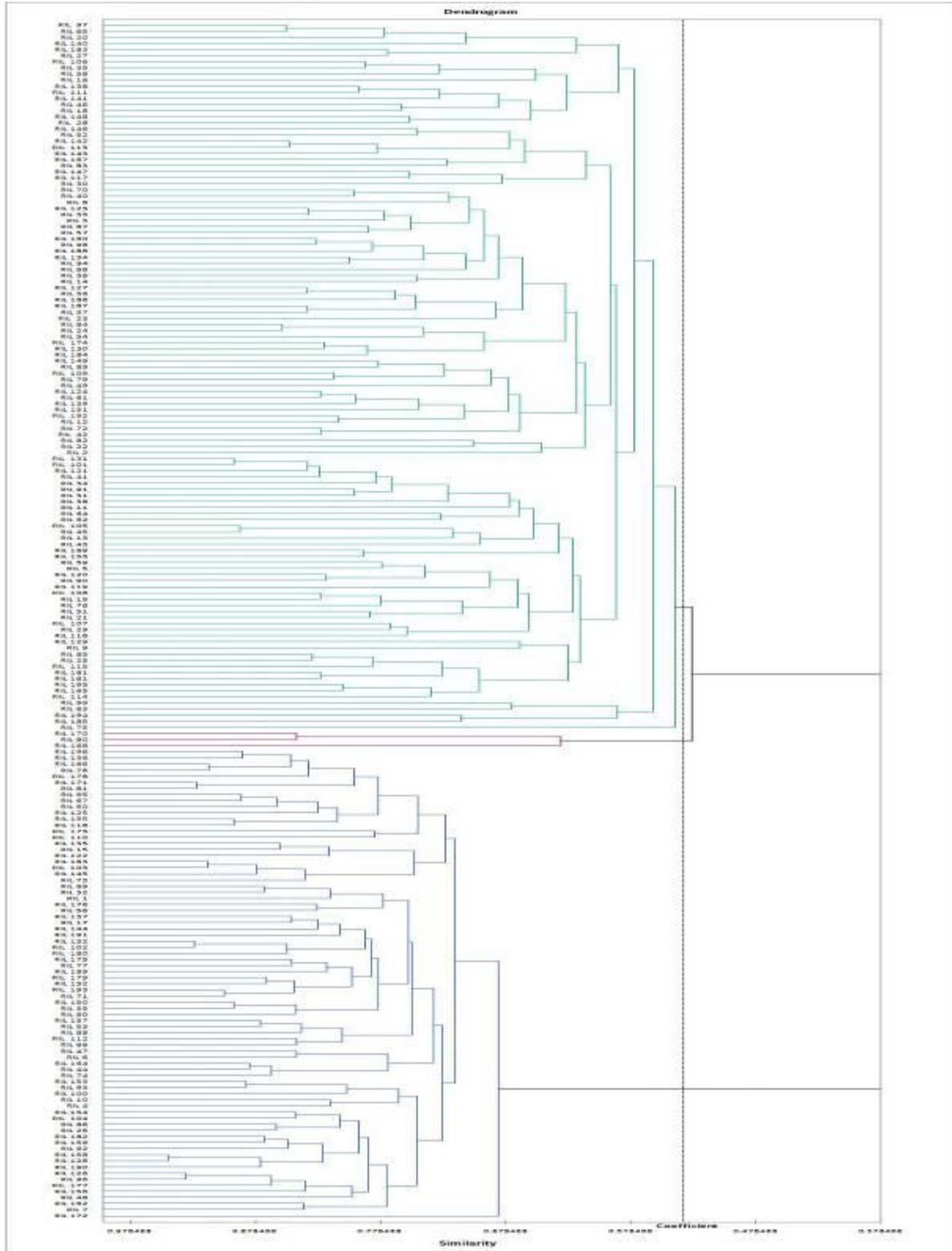




Plate.2 Screening of chickpea by Pot culture method



Plate.3 Screening of RIL's against *Fusarium* wilt by Pot culture method



Plate 4: Field view of wilt sick plot at Pulses Research Unit, Dr. P.D.K.V., Akola.

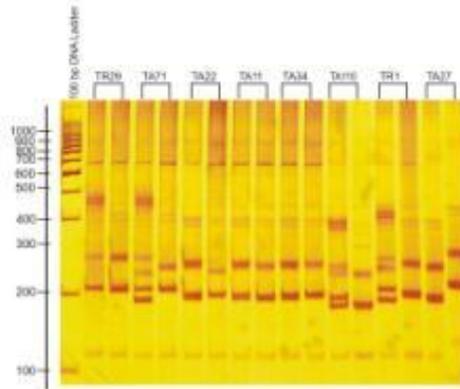
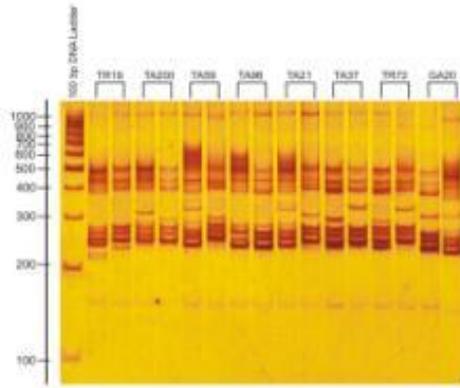
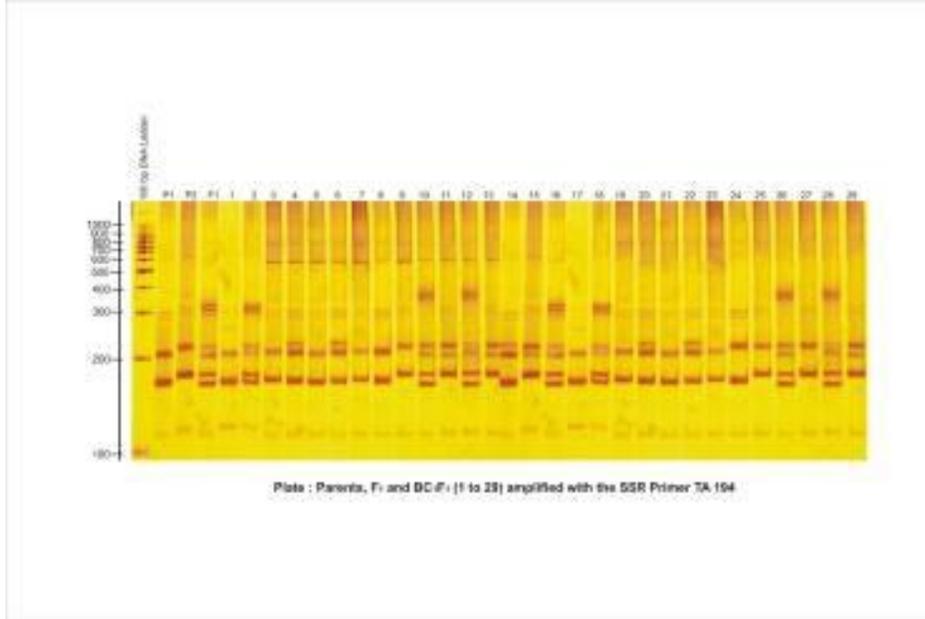


Plate : A photographic image showing parental polymorphism with SSR primers on polyacrylamide gel

coefficient 0.933 was found in between two RILs 128 and 158 that were resistant for *Fusarium* wilt indicated less genetic diversity and more genotypic similarity. Lowest similarity coefficient 0.053 was found between 133 (susceptible) and 172 (resistant) number of RILs indicated that these RILs were less similar to each other with more genetic divergence.

Dendrogram was constructed using UPGMA method of cluster analysis based on Jaccard's similarity coefficient generated by 22 SSR primers as shown in Figure 3 and 4. The dendrogram based on Jaccard's similarity coefficient could distinguish some sort of grouping among resistant and susceptible recombinant inbred lines (RILs). The dendrogram generated two main clusters at 53.62 per cent cut level of similarity. Cluster I (C₁) contained all 77 resistant recombinant inbred lines (RILs). Cluster II (C₂) contained 116 susceptible recombinant inbred lines (RILs) and 3 susceptible RIL's (RIL 80,168 and 170) were laid on same sub-cluster (C₃) of cluster II. Similar findings were reported by Padaliya *et al.*, (2013) studied six chickpea genotypes using UPGMA method.

Foreground selection for genomic regions of interest and background selection using SSR markers were employed for identification of plants for further backcrossing. Total 51 plants of the BC₁F₁ population screened with three polymorphic foreground markers (TR19, TA110 and GA16). Out of the 51 plants, only plant no. BC₁-6, 20, 26, 28, 32, 33, 40 and 41 were found to be heterozygous for three primers and plant no. BC₁-13, 17 and 50 showed heterozygous banding pattern in two primers (Table 6).

Total 51 plants of the BC₁F₁ population screened with four polymorphic background markers (TS82, TA194, TA135 and TA 22) for identifying the backcross progenies having

recurrent parent alleles. Out of the 51 BC₁F₁ plants, only plant no. BC₁-20, 33 and 41 showed background of recurrent parent for all the four markers. Plant number BC₁-28 showed background of recurrent parent in three primers (Table 7).

The wilt screening data from RIL's, BC₁F₁ and F₂ revealed segregation of a single gene with the recessive allele conferring resistance to *Fusarium* wilt. The 22 markers identified polymorphic linked markers with *Fusarium* wilt resistance trait can be effectively utilized in marker assisted selection programme and aimed towards incorporating *Fusarium* wilt resistance in chickpea. In the present study effective population size and number of markers used for marker trait correlation for *Fusarium* wilt resistance were small (i.e., only 196 RILs and 20 markers). However, the results obtained can be verified with increased population size and more number of markers.

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