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Evaluation of Gene-based Markers against Fusarium Wilt Disease in Chickpea (*Cicer arietinum* L.)

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Chickpea is an important legume crop owing to their nutritional worth. Due to the increasing population issue, it is needed to maintain the productivity of chickpeas to fulfill the requirements. At present, the main constraints in chickpea production are the Fusarium wilt disease. Forty genotypes of chickpea (*Cicer arietinum* L.) including RVG-203 as check variety were screened against Fusarium wilt resistance using six gene-based markers. Out of which five STMS markers showed polymorphism and amplified the alleles linked to resistance and susceptibility to Fusarium wilt disease in chickpea genotypes. The highest polymorphic information content (PIC) value was obtained with STMS Marker TR-29 and the least with STMS Marker TR-19. Based on molecular

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characterization, the genotypes produced similar allele as produced in the check variety RVG-203 and were identified as sources of resistance against Fusarium wilt. The results obtained in the present study open a window to use these genotypes as donor parents for the development of Fusarium wilt-resistant chickpea varieties through hybridization programs.

Keywords: Characterization; polymorphic information content; wilt; molecular markers.

1. INTRODUCTION

Chickpea (*Cicer arietinum* L.) is the 2nd most important legume crop [1] after the common bean (*Phaseolus vulgaris* L.) in the world. Chickpea consists of about 20.8% protein, 5.6% fat, 1.2% fiber, 59.8% carbohydrate, 4.8-5.5% oil, 3% ash, 0.2% calcium and 0.3% phosphorus [2]. Alongside protein, it is also wealthy in fiber, folic acid, and minerals like phosphorus, zinc, iron, calcium and magnesium. Chickpeas are a multifunctional grain legume that is widely utilized around the world, particularly as a protein source [2,3].

Chickpea production is predominantly affected by numerous biotic and abiotic stresses. Biotic stresses consist of disease - Fusarium wilt, collar rot, dry root rot, Ascochyta blight, *etc*. Among them, Fusarium wilt caused by deuteromycetes fungal pathogen *Fusarium oxysporum f.* sp. ciceris (FOC) is one of the widely disbursed diseases of chickpea [4] and cause yield loss up to the level of 100% depending on varietal susceptibility and climatic conditions [5,6]. Developing wilt-resistant chickpea varieties necessitates labour and money intensive field level phenotyping of numerous germplasm and breeding lines against pathogen races. It also takes a lot of effort. Additionally, such phenotyping using sick plots is likely to run into issues like uneven inoculum distribution and the prevalence of other soil-borne fungi [7]. In order to screen a large number of genotypes, characterising wilt resistance using established DNA markers related to wilt resistance genes is the best method.

Molecular breeding involves molecular markers for selection as well as the characterization of crop genotypes [8-10]. These markers have immense potential to increase the efficiency and precision of traditional plant breeding [11]. Genomic tools in the form of molecular markers have been developed by molecular biology to identify certain DNA variants that can be used to assist crop improvement programs [12-14]. In chickpea, various markers have been identified with their linkage to resistance genes responsible to produce resistance against different races of *F. oxysporum* [15,16]. However, it is important to use these markers for screening different chickpea genotypes to identify the source of resistance against Fusarium wilt. Considering this background, a study was performed to screen out the chickpea genotypes against Fusarium wilt disease resistance using gene-based molecular markers.

2. MATERIALS AND METHODS

2.1 Plant Material

A total of 40 chickpea genotypes (Table 1) were screened using gene-based STMS markers (Table 2) against Fusarium wilt disease at Plant Molecular Biotechnology Laboratory, Department of Plant Molecular Biology and Biotechnology, College of Agriculture, RVSKVV, Gwalior. These genotypes were collected from Jawaharlal Nehru Krishi Vishwa Vidyalaya, Jabalpur, Madhya Pradesh, India, and RAK College of Agriculture, Sehore, RVSKVV, Gwalior, Madhya Pradesh, India.

2.2 DNA Extraction

Leaf samples were collected from one-month-old seedlings from the experimental field. The collected samples were placed in cooling pads to transfer and then stored at -80 °C deep freezer. High-quality genomic DNA was extracted from 8-10 days old young and fresh leaves by employing the CTAB method as proposed by Doyle and Doyle [17] with some modifications as suggested by Tiwari *et al*. [18]. Extracted DNA was quantified through electrophoresis on 0.8% agarose gel and compared after loading a known quantity DNA marker (λ DNA) on the same gel as a standard. Apart from it, a Spectrophotometer was also used for the quantification of DNA.

2.3 Markers Analysis

The polymerase chain reaction was performed in 10 µl reaction mixture comprising of 1X PCR buffer, 0.1 µl *Taq* DNA polymerase, 1 µl dNTP (1 mM), 0.5 µl of primers (10 pM) and 20 ng/µl of

genomic DNA in a thermocycler (Bio-Rad, USA). The PCR protocol comprised of an initial denaturation step of 94°C for 3 min followed by 35 cycles of 94 °C for 1 min, annealing cycles (from 52°C to 57°C) varied for different markers system for 30 sec, elongation at 72 °C for 1 min with a final extension at 72 °C for 7 min. PCR amplified products of STMS markers along with standard markers (100 bp) was separated through electrophoresis on 3% agarose gel respectively at 100 V for two hrs. The agarose gels were stained with Ethidium Bromide (1µg/ml). After electrophoresis, the agarose gels were visualized under UV light and photographed under the Bio-Rad Gel documentation system.

2.4 Band Scoring and Data Analysis

The genetic profile of 40 chickpea genotypes was obtained based on differences in allele size using five STMS-reported markers (Table 2). The scoring was done using a standard-size ladder and a banding pattern. The data sheet was produced based on allele pattern A/A and used for further analysis. The major allele frequency, polymorphism information content (PIC), and genetic distance-based clustering were performed with Unweighted Pair Group Method for the Arithmetic average (UPGMA) tree using Power Marker v3.25 software [19].

Table 1. List of chickpea genotypes used in the investigation

S. no.	Name of genotype	S. no.	Name of genotype
1	RVSVT PS-2019- 201	21	RVSVTK-2019-106
2	RVSVT PS-2019- 202	22	RVSVTK-2019-107
3	RVSVT PS-2019- 203	23	RVSVTK-2019-108
4	RVSVT PS-2019- 204	24	RVSVTK-2019-109
5	RVSVT PS-2019- 205	25	RVSVTK-2019-110
6	RVSVT PS-2019- 206	26	RVSVTD-2019-1
7	RVSVT PS-2019- 207	27	RVSVTD-2019-2
8	RVSVT PS-2019- 208	28	RVSVTD-2019-3
9	RVSVT PS-2019- 209	29	RVSVTD-2019-4
10	RVSVT PS-2019- 210	30	RVSVTD-2019-5
11	RVSVT PS-2019- 211	31	RVSVTD-2019-6
12	RVSVT PS-2019- 212	32	RVSVTD-2019-7
13	RVSVT PS-2019- 213	33	RVSVTD-2019-8
14	RVSVT PS-2019- 214	34	RVSVTD-2019-9
15	RVSVT PS-2019- 215	35	RVSVTD-2019-10
16	RVSVTK-2019-101	36	RVSVTD-2019-11
17	RVSVTK-2019-102	37	RVSVTD-2019-12
18	RVSVTK-2019-103	38	RVSSG91-13
19	RVSVTK-2019-104	39	RVSSG96-14
20	RVSVTK-2019-105	40	RVG-203

Table 2. Details of primers used for screening of Fusarium wilt in chickpea genotypes

Primer Name	Category	Primer sequence		Reference
TA-59	STMS	F: ATC TAA AGA GAA ATC AAA ATT GTC GAA	R: GCA AAT GTGAAG CAT GTA TAG ATA AAG	[20]
TA-96	STMS	F: TGT TTT GGA GAA GAG TGA TTC	R: TGT GCA TGC AAA TTC TTA CT	[20]
TR- 19	STMS	F: TCA GTA TCA CGT GTA ATT CGT	R: CAT GAA CAT CAA GTT CTC CA	[20]
TA194	STMS	F:TTTTTGGCTTATTAGA CTGAC TT	R:TTGCCATAAAA TACAAAATCC	[20]
TR29	STMS	F:GCCCACTGAAA AATAAAAAG	R:ATTTGAACCTCA AGTTCTCG	[20]
TR31	STMS	F:CTTAATCGCACATTT ACTCTAAA ATCA	R:ATCCATTAAAACA CGGTTACCTATAA	[20]

3. RESULTS AND DISCUSSION

Due to the minimal polymorphism in the chickpea genome, the development of gene linked molecular markers has been relatively slow. The markers associated to various wilt resistance genes have been found and mapped. In the past few decades, chickpea breeders have developed an array of varieties that have performed well under field conditions. The last ten years have seen advancements in the study of chickpeas using molecular breeding tools. However, the fusion of traditional and advanced molecular breeding has accelerated the study of cereals like wheat and rice. So, similar advancements must be made in the improvement of chickpea crops to meet complete requirements. Utilizing molecular markers for the identification of desired genotypes may help in the planning of molecular breeding experiments of introgression of the targeted gene(s) in the desired genotype [21,22]. These approaches may help in deciding gene-pyramid schemes as well [23-26].

During the present investigation, 40 chickpea genotypes were evaluated with Fusarium wilt resistance gene-linked markers. Among all STMS markers only five *viz.,* TA-59, TA-96, TR-19, TA-194, and TR-29 were able to produce polymorphism in the chickpea genotypes and amplified alleles associated with resistance and susceptibility. The results found in this study are like the earlier studies. Sahu et al. [27] investigated chickpea genotypes and used gene-based molecular markers to screen them against Fusarium wilt disease. Amadabade et al. [28] investigated six chickpea genotypes, each with a distinct Fusarium wilt response, using DNA-based genetic markers associated with disease resistance/susceptibility. Padaliya et al. [29] employed seven molecular markers previously linked to disease resistance/susceptibility with six chickpea genotypes. In the present investigation, a total of 28 alleles were identified with an average of 5.6 alleles per locus for different markers (Table 3). However, Solanki et al [13] reported an average of 1.65 alleles per locus while working on diversity analysis in chickpea genotypes using different markers. Previously, Bhardwaj et al. [30] reported an average of 2.49 alleles per locus during their study on diversity assessment in chickpea genotypes using STMS markers.

The gene diversity arrayed between 0.625 to 0.790 for the markers TR-19 and TR-29 correspondingly with an average of 0.707 and Polymorphic Information Content (PIC) values varied between 0.5666 to 0.759 for the markers TR-19 and TR-29 respectively with a mean value of 0.660 respectively. The primer which showed the highest gene diversity and PIC values was TR29 while the lowest gene diversity and PIC values were detected for the primer TR-19. The major allele frequency ranged between 0.2750 (TR29) to 0.525 (TR-19) with a mean worth of 0.41.

The genetic relationships among chickpea genotypes are presented in a molecular based UPGMA tree. All the genotypes were grouped into 4 clusters. The first cluster had 9 genotypes including RVSVTD-2019-1, RVSVTD-2019-2, RVSVTD-2019-4, RVSVTD-2019-5, RVSVTD-2019-6, RVSVTD-2019-9, RVSVT PS 2019-203, RVSSG 96-14 and RVSVTD-2019-10. These genotypes are found to resemble each other at the molecular level. Further, the second cluster also contained 9 genotypes *i.e*., RVSVTK-2019-103, RVSVT PS-2019-214, RVSVT PS- 2019-213, RVSVTK- 2019- 101, RVSVT PS -2019-215, RVSVT PS -2019-104, RVSVT PS- 2019-212, RVSVTK- 2019-105along with check variety for Fusarium wilt resistance RVG-203. Grouping of these chickpea genotypes in the same cluster with the check variety (RVG-203) indicates the presence of a similar segment of DNA in them. Due to this, the applied markers were able to amplify similar banding patterns with these genotypes. A crucial step in selecting effective sources of high resilience for breeding programs is the identification of genotypes that have high stability for low disease severity. However, the presence of resistance against Fusarium wilt in chickpea genotypes grouped with the check variety RVG-203 should be confirmed before their selection as a donor in a breeding program. The job of testing breeding lines and germplasm for disease resistance is extensive and involves different methods like field trials and laboratory-based screening. However, field-level screening has few limitations because of the association of difficulties in the development and maintenance of uniform sick plots.

Consequently, the third cluster had 11 chickpea genotypes including RVSVTK-2019-109, RVSVTK-2019-108, RVSVT PS-2019-205, RVSVT PS-2019-210, RVSVT PS- 2019-204, RVSVTK-2019-102, RVSVT PS- 2019-201, RVSVT PS- 2019-202, RVSVTK-2019-110, RVSVTK-2019-106, and RVSVTK-2019-107. Forth cluster also contained 11 genotypes namely RVSVT PS-2019-208, RVSVT PS-2019-

209, RVSVT PS-2019-207, RVSVTD -2019-12, RVSSG 91-13, RVSVTD-2019-11, RVSVT PS-2019-206, RVSVTD-2019-07, RVSVTD-2019-08, RVSVT PS-2019-211, RVSTVD-2019-03. The clustering of genotypes was based on similarity indices produced on the basis of shared alleles. The genotypes grouped in the same cluster had a higher genetic similarity.

The best method for controlling chickpea wilt disease in India and other emerging nations continues to be the use of resistant cultivars. Identification of markers closely linked with Fusarium wilt resistance gene(s) is the prerequisite before performing screening of chickpea genotypes. It is important to combine field as well as laboratory-based methods in order to create linkage maps, find stable QTLs associated with Fusarium wilt resistance, and investigate novel markers in order to accurately select resistant genotypes from segregating populations. It will be easier to introduce resistant genes from Fusarium wilt-resistant cultivars carrying targeted genes into different chickpea genotypes/varieties on the availability of closely-related markers for those genes [31].

Table 3. Details of data produced by allele-specific STMS markers

Marker	Major allele frequency	Allele no.	Gene diversity	PIC
TA-59	0.400	6	0.753	0.719
TA-96	0.500	5	0.631	0.569
TR-19	0.525	5	0.625	0.567
TA-194	0.350	5	0.734	0.687
TR-29	0.2750	7	0.790	0.759
Mean	**0.410**	**5.6**	**0.707**	**0.660**

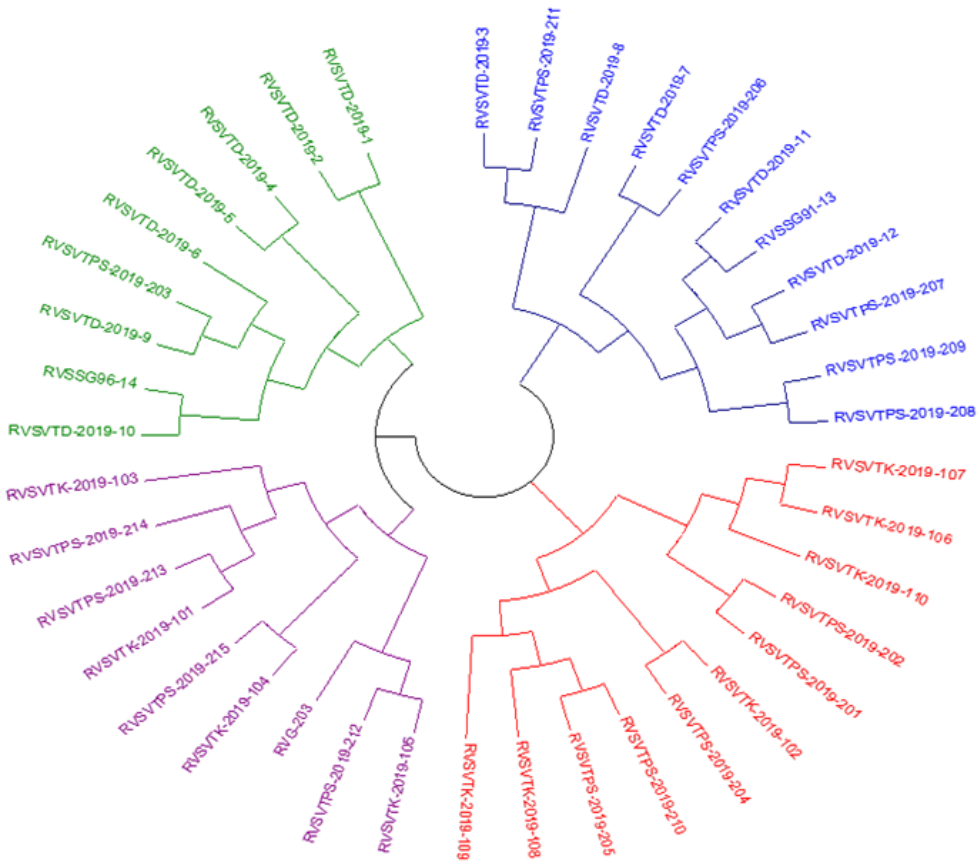

Fig. 1. Dendrogram of 40 Chickpea genotypes showing clusters based on similarity using UPGMA relationship

4. CONCLUSION

For validation of marker against Fusarium wilt in chickpea genotypes, high-quality DNA was extracted from 40 genotypes and six STMS markers were screened in selected genotypes and five markers produced polymorphism. The genotypes clustered together with the check variety RVG-203 were considered sources of resistance against Fusarium wilt in chickpeas. Resistant chickpea genotypes including RVSVTK-2019-203, RVSVT PS -2019-214, RVSVT PS -2019-213, RVSVTK-2019-101, RVSVT PS -2019-215, RVSVTK-2019-104, RVSVT PS -2019-212 and RVSVTK-2019-105 were found similar at a genomic level due to production of the same alleles using STMS markers. Resistance to these genotypes should be validated at the field level also before planning a hybridization program for chickpea improvement.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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