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RESEARCH ARTICLE

Screening of Chickpea genotypes from different agro-climatic areas against *Fusarium oxysporum* f.sp. *ciceris* (race 3) using morphological and molecular marker

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Abstract

Fusarium oxysporum f.sp. *ciceris (FOC)*, an extremely destructive pathogen, infects chickpea plants leading to over 100% losses. Although using chemicals like Carbendazim and Mancozeb the disease can be controlled but it drastically affects the soil's natural flora and fauna. Also, the emergence of new FOC races threatens the current genotypes. Many efforts have been made towards improving chickpea genotypes through breeding and selection, but the situation has not been improved over the last 2 decades. The current research uses pot screening and molecular-based approaches to screen out the resistant chickpea cultivars. In that view, the present research uses 16 chickpea genotypes collected from diverse agro-climatic areas and checked against FOC race-3. After the pot screening and ANOVA ($P<0.001$), the genotypes were categorized as highly Resistant (C 235, HC 1), resistant (GNG 2477, PHULeG 0517, GNG 2171, HC 7, PHULe G 0127), susceptible (ICCV 10) and highly susceptible (PUSA 547, RSG 931, RSG 888, ICCV 512, CSJ 513, ICCV 6). In Marker-assisted selection (MAS), the DNA of genotypes was subjected to PCR with STMS markers TA-96 and TA-27. The results revealed that the genotypes ICCV 512, C 235, GNG 2171, ICCV 10, HC 7, PHULe G 0127 and HC 1 were resistant. These results are significant for selecting resistant genotypes and can be utilized in the future validation and development of more wilt-resistant chickpea genotypes. Our results based on pot-screening and molecular-based datasets suggested a more reliable identification system for screening of FOC resistance cultivar inhibiting, which can help narrow down the selection.

Keywords

Chickpea, *Fusarium*, screening, STMS marker, pot screening

Introduction

Chickpea (*Cicer arietinum* L.) is an important leguminous crop cultivated worldwide and ranks third among the most essential leguminous crops. The crop not only has high nutritive value but is also crucial for its medicinal properties (1). In addition, being rich in proteins, carbohydrates and dietary fiber, chickpea is an inexpensive source of nutrition and therefore, is also known as Poor man's meat. The FAOSTAT data reveals that the global annual production of chickpeas is 15.87 Megatonnes (Mt) (2). Although this productivity has increased since 1961, chickpea is vulnerable to many biotic

and abiotic stresses, which has proven to be a bottleneck for the crop. One of the major stress among different biotic stresses is Fusarium wilt (FW) caused by the fungus *Fusarium oxysporum* f. sp. *ciceris* (FOC) (3, 4). FW is a highly destructive disease that leads to productivity losses of over 100% if the environmental conditions are favourable. Belonging to the class Deuteromycetes, the causal organism FOC is a very destructive pathogen that affects both the yield and fresh weight of chickpeas (5).

The literature mentions eight distinct races (0, 1A, B/C, 2, 3, 4, 5 and 6) of *Fusarium oxysporum* f. sp. *Ciceri*s that have been identified so far (6). Races 1 and 2 are predominant in central and Northern India respectively, whereas 3 and 4 are widespread in Punjab and Haryana (3). The widely adopted method of controlling the pathogen is by the use of chemical pesticides. However, non-judicial application of pesticides not only spoils soil flora and fauna but also, in the future, develop new virulent strains. Moreover, the soil-borne nature of this virulent vascular pathogen makes it challenging to be removed from the soil completely (4, 7). Therefore, the development of new races of FOC by evolution is another major factor that poses a great threat to the present chickpea genotypes (8). The adoption of tolerant cultivars is the most efficient and promising technique for disease management (1). For the development of such cultivars, a molecular-based approach of screening is required, which incorporates the use of markers. The use of DNA-based molecular markers of known sequences has an added advantage over the conventional methods of screening that they are free from any environmental influence (9). In the case of resistance against race 3, only single gene (*foc-3)* has been reported whose dominant or recessive nature is yet to be analyzed. This gene is closely linked to Sequence Tagged Micro-satellite (STMS) markers viz., and can be used for the detection of these genes (10). The STMS markers were chosen for the study because they are co-dominant, have a high level of reliability, and are easy to use (11). According to the genetic linkage map on the LG2 of Chromosome F (or G) of chickpea, there are two distinct clusters for wilt-resistant genes. Both the STMS markers TA-96 and TA-27 lie on the LG2 of chromosome and are linked with the gene *foc-3* (12, 13). The objective of the current study was thus to screen the chickpea genotypes growing in different agro-climatic conditions against *Fusarium oxysporum* f.sp. *ciceris* so that the farmers can efficiently use the resistant genotypes even on the lands infected by FOC race-3. In the future, this will help to add more disease-resistant genotypes and may open the door for the selection of wilt-resistant genotypes that will help increase the desired productivity and thus will prove economically beneficial.

Materials and Methods

Pot culture screening

The seed culture of *Fusarium oxysporum* f. sp. *Ciceris* with accession number 7679 was obtained from the Indian Type Culture Collection, IARI, Delhi, India. The initial culture was prepared by transferring the seed culture to Potato Dextrose Broth (PDB) which was then kept in a BOD incubator at 25 °C with a relative humidity of 75% for one week. To record the response of chickpea genotypes against wilt disease, a pot culture experiment was established using Completely Randomized Block Design (CRBD) at Haryana Agricultural University, Hisar, India. A total of sixteen genotypes of chickpea were collected from Haryana Agricultural University (HAU) various sources viz, including Rajasthan Agricultural Research Institute (RARI), Durgapur (Raj.) and Sri Ganganagar and were tested for disease incidence in a greenhouse (Table 1). The experimental site has a semi-arid climate in which the mean temperature recorded during November varied from 14 °C-29 °C.

Table 1. List of sixteen Chickpea genotypes with their pedigree, origin

S.No	Genotype	Pedigree	Origin
1	GNG 2477	Not available	Ganganagar, India
2	PUSA 547	Mutant of BG 256	New Delhi, India
3	PHULeG 0517	Selection from local GP	Jabalpur, India
4	RSG 931	RSG-44xRSG-524	Jabalpur, India
5	GNG 2418	GNG 1581xRSG143-1	Ganganagar, India
6	RSG 888	RSG-44xRSG-EY00Y	Jabalpur, India
7	ICC 512	Landrace from Hyderabad	ICRISAT, India
8	C-235	C-1235xIP-58	PAU, Ludhiana
9	BG 4011	Not available	New Delhi
10	GNG 2171	GNG 663 × BG 1044	Ganganagar
11	ICCV 10	P1231 × P1265	ICRISAT
12	CSJ 513	FG712 x CSJ 146	Durgapur
13	HC 7	Not available	Hisar
14	PHULeG 0127	Not available	Jabalpur
15	HC 1	Not available	Hisar
16	ICCV 6	ICC4973 X ICC4965	ICRISAT

The disease was produced by artificially inoculating the pathogen *via* seed treatment and sowing. A Neubauer hemocytometer was used to determine the spore concentration of FOC, and a suspension of 10^6 spores/ml was prepared by serial dilution (14). Seeds of all genotypes were surface sterilized and then treated with the spore suspension. Those seeds were sown in three replications in earthen pots with autoclaved soil. In control, seeds were sown in autoclaved soil only without treatment with inoculum. After the formation of seedlings, as described (15), the roots of 3 weeks old plants were dipped into the suspension culture of FOC for 15 min to expedite the process of disease infestation.

The samples showing disease symptoms were evaluated for their wilt incidence during pre and post-flowering periods using the formula:

$$\text{Wilt incidence (\%)} = (\text{Number of wilted plants/Total number of plants}) \times 100.$$

The categorization of chickpea genotypes was done according to the disease rating scale as described in Table 2.

Table 2. Disease rating scale against *Fusarium* wilt of chickpea (17)

Scale	Disease incidence	Response
1	1-10%	Highly Resistant
3	11-20%	Resistant
5	21-30%	Moderately Resistant
7	31-50%	Susceptible
9	>50%	Highly susceptible

Molecular Screening

The total DNA was isolated from the leaves of 30 days old seedlings following the CTAB method (7). The quality and quantity of extracted DNA was measured by recording absorbance at 260/280 and 260 nm respectively in a Nanodrop spectrophotometer. The intactness of DNA was checked by electrophoresis using 1% agarose gel. The STMS markers TA-97 and TA-27 (16) (Table 3), linked to wilt resistance gene *foc-3* were obtained from Eurofins Genomics India Pvt Ltd, Bangalore. The amplification by Polymerase Chain Reaction (PCR) was performed using the standard method (7); the initial denaturation was done at 94 °C for 3 min followed by 35 cycles of 94 °C for 1 min., elongation at 72 °C for 1 min with final extension at 72 °C for 10 min. The PCR products, alongside the ladder DNA, were then run on 3% agarose gel with ethidium bromide 0.5 µg/ml at 110 V for 2 hrs. The amplified PCR products were finally visualized under the Bio-Rad Gel documentation system and analysed. The genotypes were scored as resistant or susceptible based on the allele size.

Table 3. Details of STMS Primers used in the study

Name of Marker	Forward (F) and Reverse (R) sequence	Repeat motif
TA-96	F-GATAAAATCATTATTGGGTGTCCTTT	(TAA)21
	R-TTCAAATAATCTTTCATCAGTCAAATG	
TA-27	F-TGTTTTGGAGAAGAGTGATTC	(AT)3(TTA)30(AT)3
	R-TGTGCATGCAAATTCTTACT	

Disease data scoring and statistical analysis

The data collected from the screening experiment was analyzed using the mean wilting % of 3 replicates per genotype and then subjecting it to two-way ANOVA analysis with a significance level $p<0.001$. The two-way ANOVA was performed using GraphPad Prism 9.5.0 considering Pre-flowering and Post-flowering stages of wilting % and variable genotypes.

Results

The response of 16 genotypes against FOC was evaluated and analyzed using ANOVA. The wilt-resistant chickpea genotypes were identified based on pathogenicity and STMS markers specific for *foc-3*. The control plants did not exhibit any disease symptoms throughout the experiment. In contrast, the FOC-treated genotypes viz., HC 1, C235, GNG 2477, PHULeG 0127, HC 7, GNG 2171, BG 4011, GNG 2418, PHULe G 0517 showed resistance response, while the other set of genotypes viz., ICCV 6, CSJ 513, ICCV 512, RSG 888, RSG 931, PUSA 547, ICCV 10 showed a susceptible response (Fig. 1) (Table 4). The susceptible genotypes (PUSA 547, RSG 931, RSG 888, ICCV 512, CSJ 513, ICCV 10 and ICCV 6) showed a significant wilting % during pre-flowering stages whereas the resistant genotype HC 1 showed no

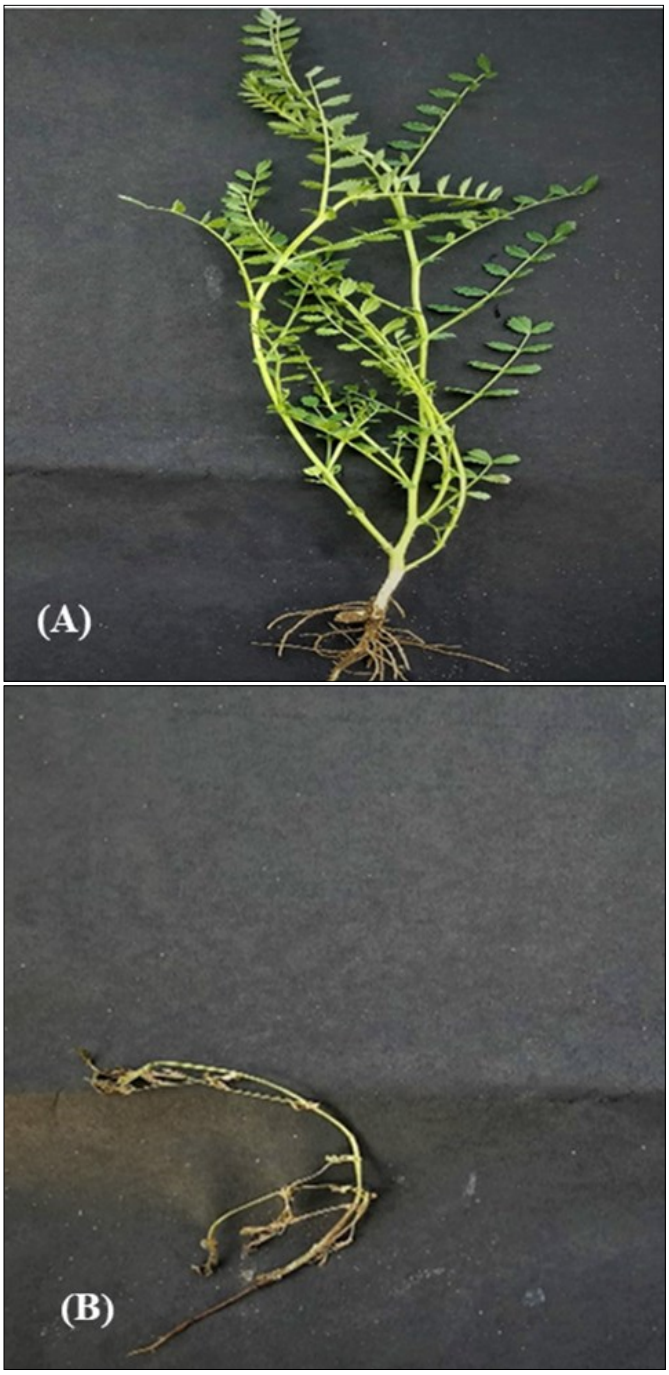

Fig. 1. A comparative image of (**A**) control plant not showing any wilting symptom (**B**) wilted plant due to FOC infection.

signs of disease during the pre-flowering stages. However, in post flowering stage, the Highly Resistant genotypes (C 235 and HC 1) showed wilting % below 10% and the resistant genotypes (GNG 2477, PHULeG 0127, HC 7, GNG 2171, BG 4011, GNG 2418, PHULe G 0517) showed a wilting % ranging between 11-20% (Table 4). The results of statistical analysis using 2-way ANOVA have been presented in Table 5. The comparative results for the wilting % in 2 stages have been represented in the graph (Fig. 2). The molecular screening of all the 16 genotypes was done

Table 4. Reaction of chickpea genotypes against *Fusarium* wilt under pot conditions

S.no	Genotype	Pre-flowering wilt %	Post-flowering wilt%	Score	Disease response
1	GNG 2477	8.78^{de}	15.08^{d}	3	R
2	PUSA 547	39.34^{bc}	79.49^{a}	9	HS
3	PHULeG 0517	3.03^{de}	11.98	3	R
4	RSG 931	53.33^{a}	87.77^{a}	9	HS
5	GNG 2418	13.55^{de}	19.84^{cd}	3	R
6	RSG 888	45.31^{ab}	74.56^{a}	9	HS
7	ICCV 512	32.32^{bc}	73.48^{a}	9	HS
8	C 235	2.38^{de}	9.11^{d}	1	HR
9	BG 4011	10.43^{de}	17.16^{cd}	3	R
10	GNG 2171	2.56^{de}	18.33^{cd}	3	R
11	ICCV 10	30.19^{c}	34.84^{c}	7	S
12	CSJ 513	38.65^{bc}	84.03^{a}	9	HS
13	HC 7	14.61^{d}	19.06^{cd}	3	R
14	PHULeG 0127	7.03^{de}	14.53^{d}	3	R
15	HC 1	0^{e}	2.77^{d}	1	HR
16	ICCV 6	36.50^{bc}	54.23^{b}	9	HS

Non-significant differences between chickpea genotypes are indicated by identical letters and were determined by Tukey HST. HR (Highly Resistant), **R** (Resistant), **S** (Susceptible), **HS** (Highly Susceptible)

Table 5. Analysis of variance (ANOVA) for wilting percentage of different genotypes at pre and post-flowering stages

Source	DF	Sum of Squares	Mean Square	F value
Genotypes	15	52553	3504***	122.2
Stages considered	1	7259	7259***	267.4
Interaction	15	5230	348.7***	12.84
Error	32	868.8	27.15	

*** represent significant values (p<0.001)

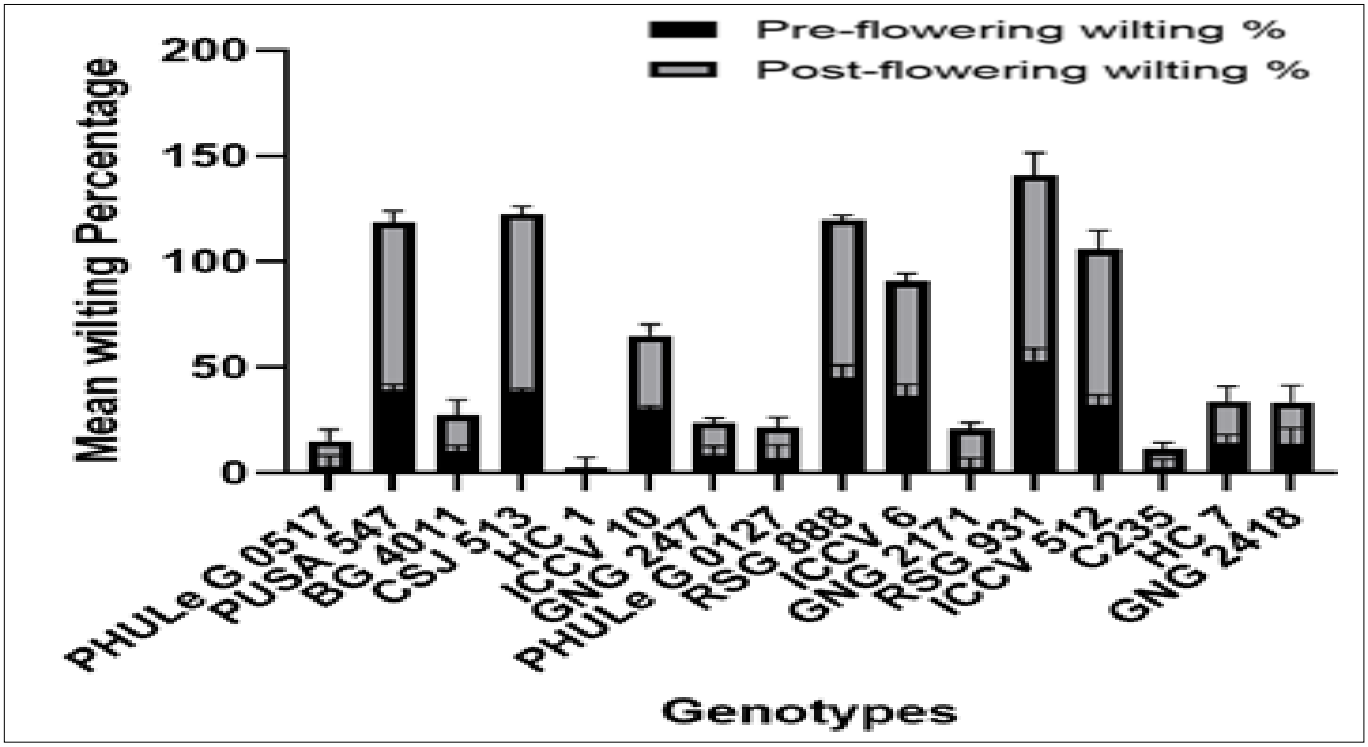

Fig. 2. Comparative wilting percentage response of chickpea genotypes against *Fusarium* infection at pre and post flowering stages.

using STMS markers TA-96 and TA-27. Based on the allelic variations of these 2 markers, the study revealed the response of these chickpea genotypes against different races of FOC. Both the markers detected polymorphism and generated 2 alleles each. The alleles of sizes 208 bp and 190 bp were generated with TA-27 (Fig. 3) for resistant and susceptible genotypes respectively. However, alleles of sizes 280 bp and 260 bp were generated for resistant and susceptible genotypes, when amplification was done with TA-96 (Fig. 4) (Table 4). The marker studies revealed that the genotypes GNG 2477, ICCV 512, C 235, GNG 2171, ICCV 10 and HC 7 were resistant to FOC, whereas PUSA 547, RSG 931, RSG 888, CSJ 513 and ICCV 6 were found to be susceptible. Two genotypes viz., PHULeG 0127 and HC 1, showed amplicons of 2 different sizes and are heterozygous for both the alleles.

experiment, the genotypes showed a wilting % ranging from 2.7-100%. These results were similar to results obtained other researchers (18) in which they found that none of the genotypes were utterly resistant to the Fusarium wilt. A significant variation between the genotypes at both of the stages was observed based on their wilting %. Various studies done in the recent past have also revealed the same differential disease reaction of the different genotypes; as observed in our case (1, 19, 20). Out of 16 genotypes, 6 genotypes (HC 1, HC 7, ICCV 10, GNG 2418, C 235, PHULe G 0517) did not show significant wilting in post-flowering stages. Whereas a significant wilting of >70% was observed in PUSA 547, RSG 931, RSG 888, ICCV 512 and CSJ 513. Results of pot screening reveal that the genotypes improved for FOC resistance (PHULe G 0517, ICCV 10) showed a low level of wilt

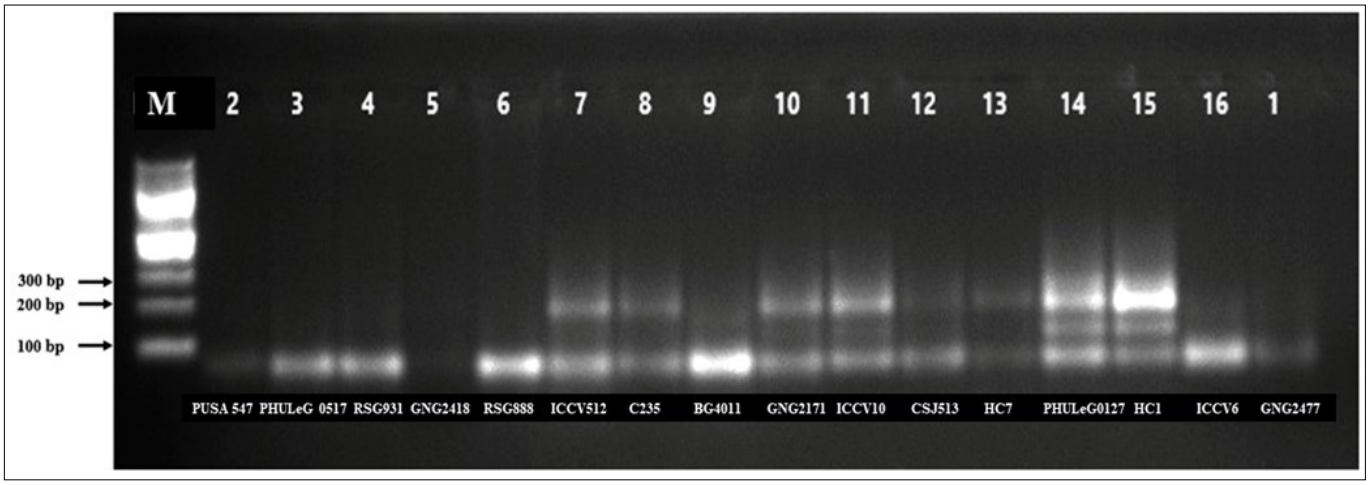

Fig. 3. Amplification of *foc-3* gene in chickpea genotypes using STMS marker TA-27 (Lane M: Molecular weight marker; 2: PUSA 547; 3: PHULeG0517; 4: RSG 931; 5: GNG 2418; 6: RSG 888; 7: ICCV 512; 8: C 235; 9: BG 4011; 10: GNG 2171; 11: ICCV 10; 12: CSJ 513; 13: HC 7; 14: PHULeG 0127; 15: HC 1; 16: ICCV 6; 1: GNG 2477).

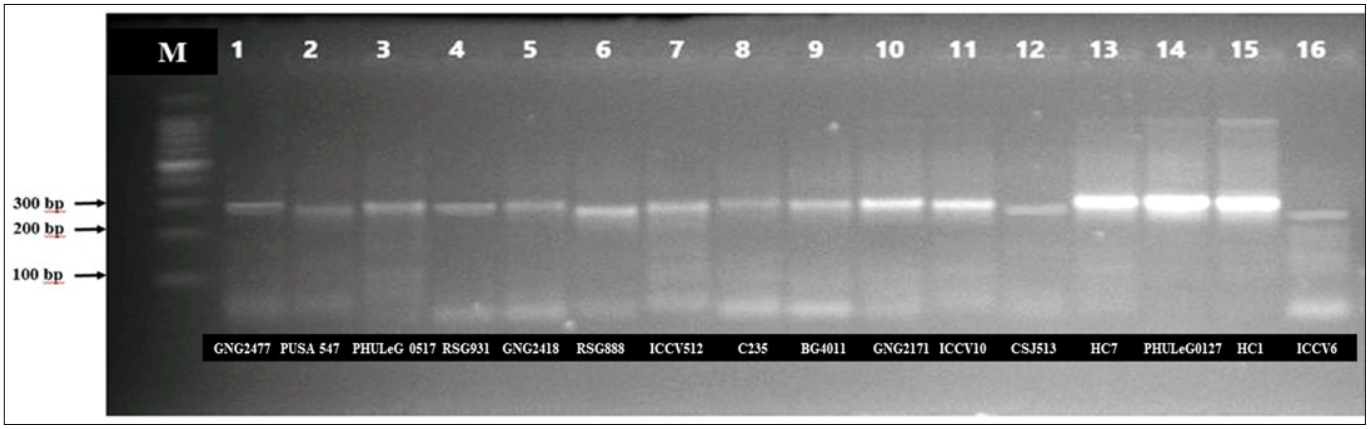

Fig. 4. Amplification of *foc-3* gene in chickpea genotypes using STMS marker TA-96. (Lane M: Molecular weight marker; 1: GNG 2477; 2: PUSA 547; 3: PHULeG0517; 4: RSG 931; 5: GNG 2418; 6: RSG 888; 7: ICCV 512; 8: C 235; 9: BG 4011; 10: GNG 2171; 11: ICCV 10; 12: CSJ 513; 13: HC 7; 14: PHULeG 0127; 15: HC 1; 16: ICCV 6).

Discussion

The plants that were infected during the pre-flowering stage during screening showed symptoms like drooping of leaves because of loss of turgor pressure in infected plants. Similar symptoms were also observed (17) and it was suggested that there was complete wilting of plants and loss of turgidity. The ANOVA analysis suggests that the plants showed a low mean wilting % (21.13) during the pre-flowering stage whereas a high mean wilting % (38.52) was observed in the post-flowering stage that reached up to 100% in the highly susceptible genotypes. In the present

incidence. On the other hand, another improved wilt-resistant genotype ICCV 6 , showed high susceptibility against race 3 of FOC. ICCV 6 was although developed as wilt resistant genotype but there are no records to support its resistivity against race-3 of FOC. A study showed that the response of genotypes PUSA 547 and RSG 888 at different FOC-infested locations varied from resistant to moderately resistant (20). However, in the present study, the genotypes PUSA 547 and RSG 888 were found to be highly susceptible. The resistance against different races of FOC is governed by distinct genes in chickpeas. Therefore, one of the reasons for the difference in the outcome in the

present study could be the race-specific resistance against the pathogen.

Thus, there is a need to develop genotypes that confer resistance against all races of FOC. Hence, molecular screening is also crucial to get ore accurate and unambiguous results. Early stage molecular screening for the selection of genotypes can thus save the time and space used in morphological screening (21, 22). The studies done previously on genetic inheritance show that the resistance to race-3 of FOC is monogenic. It has also been reported that the genes for resistance against race three have been mapped on the gene cluster CaLG02, lying closely with the STMS markers TA-96 and TA-27 (3). In the present investigation, the STMS primers TA-96 and TA-27 used to screen chickpea genotypes for FOC resistance are found to be polymorphic and generated different bands for resistance and susceptibility of genotypes. The genotypes showing FOC resistance viz., PHULeG 0517, GNG 2418, ICCV 512, C235, BG 4011, GNG 2171, ICCV 10, HC 7, PHULeG 0127 and HC 1) generated an amplified product of 280 bp while 260 bp amplification product was generated by FOC susceptible genotypes viz., PUSA 547, RSG 888 with TA-96. Some studies done in the recent past have also reported the same kind of response (22). Furthermore, TA-27 generated amplified product of size 208 bp in resistant genotypes viz., ICCV 512, C235, GNG 2171, ICCV 10 and HC 7) and amplified product of size 190 bp was generated by susceptible genotypes. Whereas, in genotypes PHULeG 0127, HC1, two alleles, both of size 208 bp and 190 bp, were observed (Table 6). These results indicate that PHULe G 0127 and HC 1 are heterozygous for resistance genes. For TA-27, no allele was observed in some genotypes (PUSA 547, PHULeG 0517, RSG 931, GNG 2418, RSG 888, BG 4011, CSJ 513, ICCV 6 and GNG 2477) whereas, for TA-96, no bands were observed in RSG 931, ICCV 6 and CSJ 513. Similar results were also observed in other studies, where no bands were observed while screening chickpeas using STMS primers, and therefore those genotypes were considered susceptible to FOC (23). PHULeG 0517, GNG 2477, GNG 2418, BG 4011 showed contrasting results when screened using markers. The resistance against FOC race 3 is monogenic, which means that the presence of any one allele is sufficient to confer resistance in the plant (3, 26). Thus, the presence of markers associated with either TA-96 or TA-27 suggest that the genotype is resistant against FOC. These results are also supported by the pot-screening experiments.

Although the molecular screening done in the present study supports most of the pot screening results, the response of genotype ICC 512 and ICCV10 was found to be contrasting. The genotype ICCV 10 was released for the central and southern zones of India. ICC 512 on the other hand is a landrace acquired from Hyderabad (25, 26). These areas have a significantly different climatic and soil conditions from the Northern zone where the experiment was carried out. Thus, this difference in results may be due to a significant difference in the climatic conditions.

As a result, the current work demonstrates that these 2 STMS primers may be used well for large-scale screening of chickpea genotypes in disease resistance breeding, and therefore for a marker-assisted breeding programme.

Conclusion

The interest of researchers in chickpeas has increased over the past few years, and marketers are searching for genotypes that can give them high productivity alongside being wilt-resistant. A constant and timely screening of chickpea genotypes is necessary because of the continuous

Table 6. Alleles generated by STMS markers TA-96 and TA-27 and their response to FOC based PCR results

S.No.	Genotype	Allele generated with marker TA-96	Disease response	Allele generated with marker TA-27	Disease response
1	GNG 2477	280 bp	Homozygous Resistant	No clear band	Susceptible
2	PUSA 547	260 bp	Homozygous Susceptible	No clear band	Susceptible
3	PHULeG 0517	280 bp	Homozygous Resistant	No clear band	Susceptible
4	RSG 931	No clear band	Susceptible	No clear band	Susceptible
5	GNG 2418	280 bp	Homozygous Resistant	No clear band	Susceptible
6	RSG 888	260 bp	Homozygous Susceptible	No clear band	Susceptible
7	ICCV 512	280 bp	Homozygous Resistant	208 bp	Homozygous Resistant
8	C 235	280 bp	Homozygous Resistant	208 bp	Homozygous Resistant
9	BG 4011	280 bp	Homozygous Resistant	No clear band	Susceptible
10	GNG 2171	280 bp	Homozygous Resistant	208 bp	Homozygous Resistant
11	ICCV 10	280 bp	Homozygous Resistant	208 bp	Homozygous Resistant
12	CSJ 513	No clear band	Susceptible	No clear band	Susceptible
13	HC 7	280 bp	Homozygous Resistant	208 bp	Homozygous Resistant
14	PHULeG 0127	280 bp	Homozygous Resistant	208 bp, 190 bp	Heterozygous Resistant
15	HC 1	280 bp	Homozygous Resistant	208 bp, 190 bp	Heterozygous Resistant
16	ICCV 6	No clear band	Susceptible	No clear band	Susceptible

evolution of new races and pathotypes of FOC. The prevalence of race 3 of FOC in Punjab makes the present study significant as it will help identify the chickpea genotypes that can effectively resist the *Fusarium* wilt. Based on both pot and molecular screening result analysis, it can be concluded that the genotypes HC 1, HC 7, PHULe G 0127, C235, GNG 2477, PHULe G 0517, GNG 2418, BG 4011 and GNG 2171 are resistant to FOC race-3 and therefore, can be effectively used by the farmers in the areas infected with FOC race 3. Thus, this pot experiment, along with MAS, can help in reducing and eliminating the common occurrence of ambiguous results, thus helping in narrowing down the selection of the most resistant and susceptible cultivars.

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Authors contributions

S carried out the research work and drafted the manuscript. SK contributed in the statistical analysis and paper. SK helped in alignment and editing of the manuscript. VG provided all the resources, work place required to carry out the whole research work.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interests to declare.

Ethical issues: None.

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