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BRIDGING THE CONVENTIONAL AND MOLECULAR METHODS FOR IDENTIFICATION OF RESISTANCE AGAINST *FUSARIUM* WILT IN CHICKPEA GERMPLASM

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ABSTRACT

Chickpea is an economically important food crop and its production is affected by many biotic and abiotic factors. One of the common pathogens among root rot and wilt is *Fusarium* in chickpea producing regions of the world which causes yield loss upto 10% to 40%. In this study twenty eight chickpea genotypes were analysed for resistance against *Fusarium* caused by *Fusarium oxysporum* f.sp. *ciceris* wilt using both conventional and molecular methods to identify source of resistance. In conventional method, 28 chickpea germplasm lines were tested for effect of *Fusarium oxysporum* (*Foc) belonging to race 2* in a completely randomized pattern. After about four weeks of inoculation the resistance and susceptibility of plants to *Foc* was obvious. In molecular analyses all twenty eight genotypes were screened with primers TA110, Tr19, Ts82, TA194, TA27 and TA96 and CS27A which are closely linked markers with *Foc* 2 resistance gene (Accession No. FJ538241). In eighteen of the genotypes the results of conventional study was found in accordance with molecular analysis. Marker assisted selection may be helpful in screening of available chickpea genotypes for wilt resistant genes. Chickpea genotypes identified as resistant during this study may be exploited in breeding programs to develop *Foc* race 2 resistant germplasm.

Keywords: Chickpea, *Fusarium* wilt, resistant genes, breeding.

INTRODUCTION

Chickpea (*Cicer arietinum* L.*)* is one of the important annual grain legume in various countries. It is grown in the arid and semi dry areas of the world. It is rich in protein contents and hence regarded as highly nutritional food. In Pakistan, chickpea is grown on 82% area in Punjab, 9% of NWFP, 8% of Sindh while 1% area in Baluchistan. The main chickpea producing area in Pakistan is Thal desert which contributes about 80% to its production. Thal is considered as the home of chickpea crop in Pakistan (Khan *et al*. 1991). There is an immense need to maintain and improve its yield (Sivaramakrishnan *et al*. 2002; Ahmad *et al*. 2014).

Different biotic and abiotic stresses cause yield losses in chickpea, the most destructive biotic stress among them are fungal diseases. Until now above 50 different types of pathogens had been studied in chickpea from diverse part of the globe. Some of them are sources of serious economic losses including *Fusarium oxysporum* f. sp. *ciceris* (*Foc*) causing *Fusarium* wilt. Among root rot and wilt pathogens, *F. oxysporum* is common in chickpea producing regions of the world and causes loss between 10% to 40% (Nene *et al*. 1984; Kaiser *et al*. 1994). *Fusarium* wilt caused by *Foc* is the main concern amongst the biotic factors and has a worldwide occurrence (Jalali and Chand, 1992). *Foc* has eight pathogenic races (races 0, 1A, 1B/C, 2, 3, 4, 5 and 6). Races 1A, 2, 3, 4, 5 and 6 cause wilting symptoms in susceptible chickpea cultivars, but races 0 and 1B/C cause yellowing. Races 0, 1A, 1B/C, 5 and 6 are established generally in the Mediterranean area and California (Jimenez-Gasco *et al.* 2004). Race 5 is the largely active in Spain (Landa *et al.* 2004). Race 6 is the most frequent after race 0 (Jimenez-Diaz *et al*. 1989). Whereas in Pakistan race 2 from Thal area whereas a biotype of race 6 was identified from Thar and Faisalabad regions based on DNA markers and conventional data (Ali, 2014).

Fusarium wilt of chickpea is seed-borne and infected seeds when mixed with healthy seeds can carry the fungus to new areas and can set up the disease into the soil to cost effective threshold levels within three chickpea growing seasons (Pande *et al*. 2007). This disease can be controlled by chemicals, biological and screening methods. Chemical control of wilt is not much efficient and economical because the pathogen is soil borne as well as seed borne and hard to eliminate. Fungal spores can live in soil up to six years even in the lack of the host plants (Haware *et al*. 1996). Identification of resistance to biotic and abiotic stresses as well as agronomic traits using molecular markers and allele

mining is a promising approach (Upadhyaya *et al*. 2011; Das *et al*. 2015). Marker assisted selection (MAS) supported by the utilization of DNA markers linked to wilt resistance genes can be employed to differentiate a large numbers of chickpea genotypes. On the other hand conventional screening of a large number of germplasm for resistance to all races of the pathogen is expensive, time consuming and difficult due to required inoculum and environmental conditions (Tullu *et al.* 1998; Gupta and Varshnay, 2008).

In earlier studies, a number of DNA markers (TA194, Tr19, H1B06, TS82, CS27A, TA27, TA59, TA96, H1F22, H1P09/2 H1F22, H1P09/2, TA103, TA110, H1F05, and H6D11) were mapped on Linkage Group 2 (LG2) of the chickpea map (Winter *et al*. 2000; Sharma *et al*. 2004; Cobos *et al.* 2006). *Fusarium* wilt resistance genes have been mapped on LG2, and these markers are closely linked to the genes conferring *Fusarium* wilt resistance (Cobos *et al*. 2006). This study was designed to evaluate the chickpea germplasm for resistance against local isolate of the *F. Oxysporum,* race 2 (data not published) using molecular markers and conventional methods to compare the validity of both methods. We have used previously mapped markers to indicate *Fusarium* wilt resistance genes within our local germplasm. The objective of this study was to link markers to both susceptibility and resistance alleles.

MATERIALS AND METHODS

The present work was performed at the Nuclear Institute of Agriculture and Biology (NIAB), Faisalabad and department of Bioinformatics and Biotechnology, GC University, Faisalabad.

Plant material: Seeds of twenty eight genotypes were comprised of eight parents (Table 1) and their twenty induced mutants and hybrids (Table 2). The germplasm was developed at NIAB. Seeds of these genotypes were sown in autoclaved sand pots.

Conventional screening for *Fusarium oxysporum*

Inoculum preparation: Inoculum of the *F. oxysporum* f. sp. *ciceri*s (*Foc*) race-2 was prepared from 21 days old cultures grown in Kerr's media broth (Kerr, 1994) under invariable cool fluorescent light at 25°C with continuous shaking at 100 rpm. From the cultures the mycelium was separated by grinding the cultures in blender and passing through mesh. Spores concentration was adjusted 1×10^6 per ml with the help of haemocytometer. Chickpea genotypes were sown in autoclaved sand pots. At three to four leaf stages i.e.10 days old seedlings were uprooted and with the help of sterile scissors and lower one fifth of the roots tips were trimmed. These seedlings were dipped into spore suspension of *Foc 2* for 5 minutes and transferred into another autoclaved soil and clay potting mix. By three replications in a completely randomized pattern, the genotypes were analysed for effect of the race 2 following the method utilized by Sharma *et al.* (2005). In a similar way non inoculated control plants were treated except for that their trimmed roots were dipped in autoclaved distilled water for about 5 min then transferred into other autoclaved soil and clay potting mix. The plants were grown in growth room conditions with a temperature range of about 22 and 26°C (12 h: 12 h) and photoperiod for growth of plants was fluorescent light for 16 hours.

Diseases assessment and data analysis: Disease frequency was recorded after one week of inoculation and at weekly intervals for 4 weeks. Using the following formula the wilt occurrence of each genotype was determined.

$$\text{Wilt Incidence} = X/Y*100$$

(where "X" is number of plants wilted while "Y" represents Total number of plants sown).

Using the disease rating scale of Sharma *et al.* (2005) the level of resistance and susceptibility of each tested genotype was determined. The disease frequency data was analysed and converted into definite data by using the following way: 0 to 10% wilting is resistant, 11 to 89% wilting is intermediate and >90% wilting is susceptible according to disease incidence score of Sharma *et al.* (2005).

Molecular method for screening *Fusarium oxysporum*

DNA extraction: DNA from 10 days old seedlings was extracted following a modified CTAB method (Khan *et al*., 2004). The DNA concentration of 28 genotypes was quantified with the help of a spectrophotometer and the concentration was adjusted to 10ng/ul for PCR analysis.

Sequence tagged microsatellite sites (STMS): A total of 7 primers including six sequence tagged microsatellite (STMS) primers and one ASAP (allele specific associated primer) were utilized for the genotyping. The ASAP marker CS27 which has been mapped on LG2 of both intraspecific and interspecific chickpea maps was selected (Winter *et al*. 2000; Tekeoglu *et al.* 2002; Udupa and Baum, 2003).

PCR profile for ASAP primer: ASAP primer pair CS-27F and CS-27R was synthesized from Gene Link (USA). Using the MyGene MG 96 G gradient thermal cycler the amplification reaction was carried out.

STMS (PCR) profile: Six STMS markers (TA110, Tr19, Ts 82, TA194, TA27 and TA96) previously reported for chickpea resistance to different races of *Foc* were used in this study. The PCR amplification reaction volume was 15 µl, consisting of 0.2 mM of dNTPs, 50 ng DNA, 0.8% Nonidet P_{40}, 2.5 mM $MgCl_2$, 0.8 U Taq DNA polymerase and 0.35 µM of each primer. Thermal cycler was programmed as following, for the first step of

denaturation at 94°C for 2 minutes, followed by 35 cycles denaturation of 20 second at 94°C, annealing for 50 seconds at 55°C, and extension for 50 seconds at 60°C.

Agarose gel electrophoresis and PAGE for DNA markers: The PCR products were electrophoresed at 120 V in 8% polyacrylamide gel electrophoresis (PAGE) for approximately two hours using 0.5X TBE buffer alongside a DNA molecular size marker. Acrylamide and agarose gel were stained with ethidium bromide; photographs were made by UVI Pro Platinum System. Then analysis of the gels was completed by using UVI BandMap 1.1 tool.

RESULTS AND DISCUSSION

Chickpea is of great interest to legume breeders. Many varieties have been developed, having various economically important characteristics for the last several decades. In a decade or so, a great progress has been done in chickpea biotechnology. The genotypes are being screened using molecular markers to be used in gene pyramiding and other breeding programs. Screening of wilt resistant genes in chickpea can be carried out by using advance biotechnological tools. Marker Assisted Selection is one of the various tools which can be used to accelerate conventional breeding (Ravikumar and Babu, 2007). There are 8 diverse races of *fusarium* that cause wilt in chickpea. More than one gene is involved in resistance mechanism (Hu *et al.* 2015). Genetic stocks should be developed for all of them as a source for complete and composite genetic resistance. Tagging of *fusarium* resistance genes through closely related co-dominant and dominant DNA markers are efficient methods in identification of wilt resistant genes of chickpea.

Identification of *Fusarium* wilt resistant chickpea genotypes was done on the basis of pathogenicity and STMS markers of chickpea genotypes. Disease percentage of twenty eight chickpea genotypes against *Foc* 2 was evaluated (Table 3). Throughout the experiments control plants did not exhibit any wilt symptoms. All the susceptible genotypes that were inoculated with *Foc* 2 had shown disease symptoms after about 2 weeks of inoculation and died after four weeks. The four genotypes (97086, CH85/06, CM562/05 and CM526/05) illustrated expected results of resistant for the *Foc* 2 (Table 3).

Phenotypic response to Foc 2: In present study twenty eight chickpea genotypes have been used to find resistant genotypes against *Fusarium* wilt by both conventional and molecular methods. The 28 genotypes were tested for local isolate of *F. oxysporum* race 2 (*Foc2*) with three replications of each genotype in a completely randomized pattern. After about four weeks of inoculation with *Foc* 2, all plants of P1-13, PUSA329, CM739/06, CH120/04 and Noor91 wilted and died, while 97086, CH85/06, CM562/05, CM526/05 survived and showed resistance. The resistance in chickpea germplasm has also been reported by other workers against *Fusarium* wilt (Sharma *et al.* 2004; Chaudhry *et al*. 2006; Intizar-ul-Hassan *et al*. 2011). In a study, one hundred twenty two germplasm lines against *fusarium* wilt were evaluated and 37 out of them were found to be resistant, although all the other germplasm lines were moderately resistant to greatly susceptible. Chaudhry *et al*. (2006) screened 414 germplasm accessions for *Fusarium* wilt and found 35 lines resistant, 208 intermediate, 77 susceptible and 94 highly susceptible.

The 28 germplasm lines were tested for effect of *Foc* 2 in three replications of each in a completely randomized design. After about four weeks of inoculation, all plants of P1-13, Pusa329, CM739/06, CH120/04 (Figure 1) and Noor91 wilted and died latterly, four genotypes namely 97086, CH85/06, CM562/05 and CM526/05 survived and showed resistance according to phenotypic analysis. Resistance in chickpea for race 2 of *Fusarium* wilt was estimated according to the classification of Sharma *et al*. (2005). The late wilting chickpea genotypes also show 100% wilt incidence with delayed set of symptoms and fit well into the suggested classification. Slow wilting genotypes are included into the intermediate group. Because of environmental variation and experimental errors a margin of 10% variability has been kept (Sharma *et al*. 2005).

Survey of SSR and ASAP markers for chickpea: The twenty eight genotypes were screened with six primers, out of these, the polymorphic pattern by three SSR markers (TA27, TA96 and TA194) did not correlated with our phenotypic study while the other three (TA110, Tr19, TS82) correlated well with our phenotypic study (Fig.3) which have been mapped on linkage group 2 (LG2) in both inter and intraspecific crosses (Mayer *et al*. 1997; Winter *et al*. 2000; Tekeoglu *et al*. 2002; Udupa and Baum, 2003) and were found to be closely linked with *Foc* 2 resistance. In molecular analysis six genotypes were found to be resistant to *Foc* 2 with all the seven primers used. For the marker TA110 susceptible genotypes were also resolved. TA110 showed 8 of the 28 genotypes as resistant to *Foc* 2. The genotypes CH85/06, 97086, CM156/05, CM573/04, CM562/05, CM526/05, CH120/04 and Bittle98 were found to carry resistance allele for TA 110 marker.

All the twenty eight genotypes were also screened with primers TA110, Tr19, TS82 and CS27 (Winter *et al*. 2000; Tekeoglu *et al*. 2002; Udupa and Baum, 2003) which were found to be closely linked with *Foc* 2 resistance genes. Three of the resistant genotypes 97086, CM562/05, CM526/05 were found resistant with both conventional and all the four markers used which has validated the results. Six of the genotypes which were

found susceptible with conventional method CM601/06, CM739/06, CH 81/06, CM709/06, PUSA329, Pb1 were also validated by all the four primers used. In a previous study, 14 of 16 genotypes that were R or S to *Foc*1, *Foc* 2 and *Foc* 3 were correctly verified as R and S through TA110 marker (Gowda *et al*. 2009). Ravikumar and Babu (2007) had also used these markers to understand the inheritance pattern of *Fusarium* wilt resistant alleles in various chickpea genotypes.

The earlier reported DNA markers were useful to check their relationship with phenotypic data and their association with the resistant genes to *Foc* 2. Molecular markers utilized for this purpose included diverse STMS markers (Sharma *et al*. 2004 and 2010) as well as ASAP marker ($CS27_{700}$) (Mayer *et al.* 1997; Soregaon and Ravikumar, 2010). Due to minimum polymorphism in chickpea genome, implementation of molecular markers had been moderately slow (Kazan and Muehlbauer, 1993). In case of Tr19, the resistance allele for *Foc* 2 was present in genotypes 97086, CM156/05, CM573/04, Bittle 98, CM526/05, CM1004/06, 96052, CH120/04 and CM562/05. In the same way for CS27 which is linked with susceptibility, the allele linked with susceptibility was missing in P1-13, 97086, CM156/05, CM573/04, CM562/05, CM526/05, CM759/06 and CM956/06 and was present in all other genotypes. For Ts 82, *Foc* 2 resistant genotypes were CH120/04, CM1004/06, 96052, 97086, CM156/05, CM573/04, CM562/05 and CM526/05.

Interestingly, the six genotypes, which shown resistance against *Foc* 2 with TA110, also exhibited resistance with the Tr19, CS27 and TS82 primers surveyed. Three of the genotypes 97086, CM562/05 and CM526/05 which were found resistant with conventional methods were also found having resistance alleles with all the four markers used. There are some controversies as well that the genotypes 96052 and CM1004/06, which came out *Foc*2 resistant, were found susceptible with TA110 and Cs27 primer. The genotypes P1-13, CM759/06 and CM956/06, which appeared *Foc* 2 resistant with Cs27 primer, were found susceptible with TA110, Tr19 and TS82 because Cs 27 is more closely linked with race 1.

While CH85/06 was found resistant by conventional method and two of the markers validated its resistance. The genotypes CM156/05 and CM573/04 were found intermediate with the conventional methods but all the four markers showed resistance alleles in them. Six genotypes CM601/06, CM739/06, CH 81/06, CM709/06, PUSA329, Pb1 were found susceptible with both conventional and all the four markers used. On total in eighteen of the genotypes conventional and molecular methods showed compatible results while in ten genotypes there was controversy in the results.

The resistant lines with desirable agronomic traits and appreciable yield potential could either be released directly as commercial cultivars or they may be used as source of resistant genes to transfer into commercial cultivars lacking resistance, through conventional breeding procedures. However, prior to such transfer of their resistance to a commercial cultivar the genetic basis of resistance must be determined against the virulence of *F. oxysporum* (Bajwa *et al.* 2000).

To understand resistance mechanism and genes involved in disease resistance molecular markers have been recognized as a powerful tool. Close linkage of microsatellite markers with resistance genes has been recognized for many other crops as well and the traits like the bacterial leaf blight in rice (Blair and Mccouch, 1997), *Fusarium* wilt resistance genes in chickpea (Gowda *et al*. 2009) and soybean cyst nematode resistance in *Glycine soja* (Shawn *et al*. 2007). Resistance to *Foc* races 1, 2 and 3 was previous administrated by 3, 2 or 1 gene, correspondingly (Sharma *et al*. 2004, 2005). Mayer *et al*. (1997) described first the linkage of the marker CS27 with *Foc* 1 at 7.0 cM and afterwards this marker was modified into an allele specific related marker (CS27A). Sharma *et al*. (2004) found that *Foc* 2 resistance gene was located near two SSR markers H3A12 and TA96, at an interval of 2.7 cM and 0.2 cM correspondingly.

This study has validated that the DNA markers can be used in MAS to speed up conventional breeding (Ravikumar and Babu, 2007; Ahmad *et al.* 2014). To develop resistant varieties, Chickpea genotypes identified as resistant during this study may be utilized into breeding programs. Effectiveness of MAS, On the other hand, depends upon closeness of the markers with the genes. The cheapest, economical and the most ideal way of managing chickpea wilt, is to develop resistant cultivars and breeders can interpret conventional and molecular approaches to achieve this goal. The resistant germplasm would be a good source for chickpea breeders. Resistant germplasm would increase average yield of chickpea. The existing chickpea genotypes could be used for genome mapping and gene tagging for favourable traits.

Table 1. Parental chickpea genotypes used in this study.

S. No.	Parents	Types
1.	Bittle-98	Desi
2.	Line-96052	Desi
3.	Noor-91	Kabuli
4.	Line-97086	Desi
5.	Pusa-329	Desi
6.	CM-2008	Kabuli
7.	PI-13	Desi
8.	Pb-1	Kabuli

Table 2. Pedigree of chickpea hybrids and induced mutants used in this study.

S. No.	Mutants	Pedigree	Desi/Kabuli
1.	CH120/04	96052xBittle98	Desi
2.	CC121/00	Pb-1	Kabuli
3.	CH73/02	Noor 291xBittle98	Kabuli
4.	CM156/05	97086 (induced mutant 50kR)	Desi
5.	KCM573/04	97086 (induced mutant 60kR)	Desi
6.	CM601/06	CM2008 (induced mutant 25kR)	Kabuli
7.	CM739/06	CM2008 (induced mutant 25kR)	Kabuli
8.	CM759/06	CM2008 (induced mutant 25kR)	Kabuli
9.	CM770/06	CM2008 (induced mutant 25kR)	Kabuli
10.	CM956/06	CM2008 (induced mutant 35kR)	Kabuli
11.	CH81/06	PI-13xPassa329	Desi
12.	CM562/05	97086 (induced mutant 60kR)	Desi
13.	CH85/06	PI-13*Passa329	Desi
14.	CM526/05	97086 (mutant 50kR)	Desi
15.	CH91/06	PI-13xPassa329	Desi
16.	CM709/06	CM2008 (induced mutant 25kR)	Kabuli
17.	CM1004/06	CM2008 (induced mutant 35kR)	Kabuli
18.	CM1012/06	CM2008 (induced mutant 35kR)	Kabuli
19.	CM888/06	CM2008 (induced mutant 25kR)	Kabuli
20.	CM877/06	CM2008 (induced mutant 25kR)	Kabuli

Table 3. Relationship of markers for resistance and phenotype between selected local germplasm for race 2 of *Fusarium oxysporum*.

S. No.	Genotypes	Phenotypic	Data	TA110	Tr19	CS27A	Ts82
1.	CH120/04	100	S	R	R	S	R
2.	CC121/00	66	I	S	S	S	S
3.	CH73/02	60	I	S	S	S	S
4.	CM156/05	53	I	R	R	R	R
5.	CM573/04	40	I	R	R	R	R
6.	CM601/06	80	S	S	S	S	S
7.	CM739/06	100	S	S	S	S	S
8.	CM759/06	66	I	S	S	R	S
9.	CM770/06	73	I	S	S	S	S
10.	CM956/06	73	I	S	S	R	S
11.	CH 81/06	83	S	S	S	S	S
12.	CM562/05	10	R	R	R	R	R
13.	CH85/06	13	R	R	S	R	S
14.	CM526/05	13	R	R	R	R	R
15.	CH91/06	50	I	S	S	S	S
16.	CM709/06	87	S	S	S	S	S
17.	CM1004/06	87	S	S	R	S	R
18.	CM1012/06	73	I	S	S	S	S
19.	CM888/06	66	I	S	S	S	S
20.	CM877/06	66	I	S	S	S	S
21.	Bittle 98	73	I	R	R	S	S
22.	96052	60	I	S	R	S	R
23.	Noor91	100	S	S	S	S	R
24.	97086	13	R	R	R	R	R
25.	PUSA329	100	S	S	S	S	S
26.	CM2008	60	I	S	S	S	S
27.	Pb1	87	S	S	S	S	S
28	P1-13	100	S	S	S	R	S

I = Intermediate, S = Susceptible, R = Resistant

Figure 1. Plants of CH120/04 wilted dropped and finally died, control plants remained unwilted

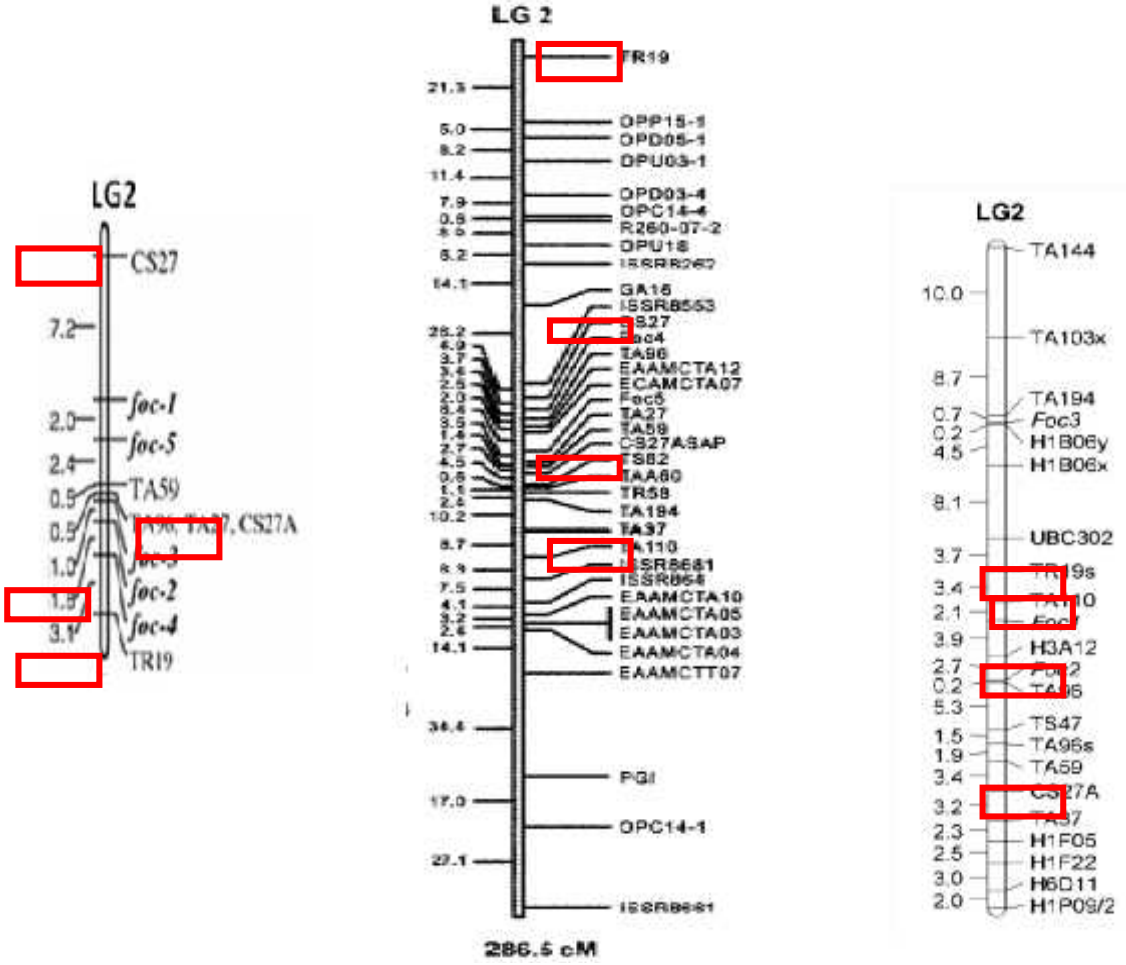

Figure 2. Linkage map of chickpea developed by interspecific (Winter *et al.*, 2000) and intraspecific crosses

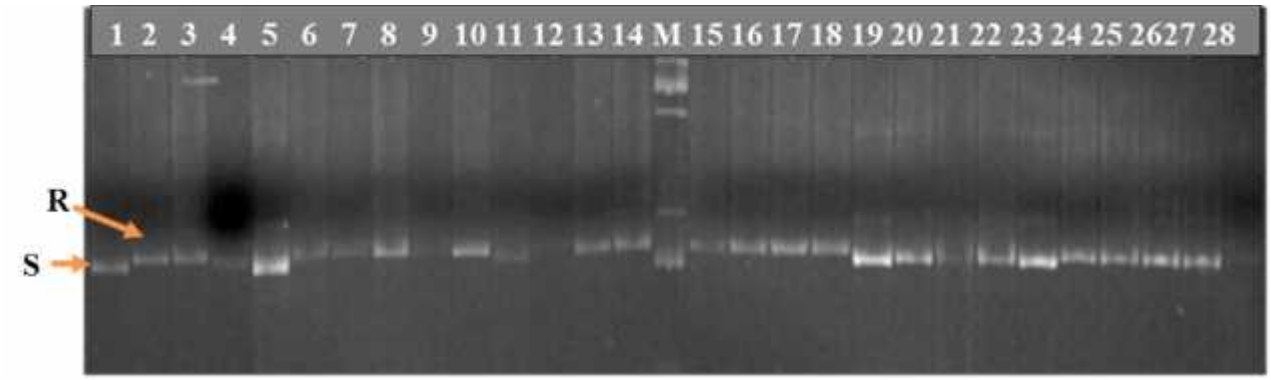

Figure3. STMS-PCR of Chickpea genotypes with primer Ts 82. M is 1Kb ladder. Lane 1-28 are the chickpea genotypes used in this study.

Conclusions: Marker Assisted Selection (MAS) coupled with other biotechnological tools is a strong source to understand various economically important phenomenon in today's agriculture. The modern technologies can bridge the conventional methods to solve the major problems. In this research project, we identified the fusarium wilt linked genes in chickpea by using different markers. Total of 28 genotypes of chickpea were screened for the disease after inoculation. All the markers were found linked with *Foc2* gene which is wilt resistant. The information obtained in this study may help in future MAS projects of chickpea breeding.

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