A genetic linkage map of the chickpea, *Cicer arietinum*, based on microsatellite markers and localization of the Fusarium vascular wilt resistance locus

(Received: 26. 04. 2018; Accepted: 20.05.2018)

Fouad N. [1], Hamwieh A. [1], Abdel-Hamied A. [2], Saad M. E. [2, 4] Youssef M. M. [3]

[1]Biodiversity and Integrated Gene Management Program (BIGM), The International Centre for Agricultural Research in the Dry Areas, (ICARDA), Giza, Egypt.

[2]Department of Nucleic Acid, Proteomics and Genomics, Genomics facility Central Lab, Agricultural Genetic Engineering Research Institute (AGERI), Agricultural Research Center ARC, Egypt.

[3]Department of Chemistry, Faculty of Science, Cairo University, Giza, Egypt.

[4]Department of Biology, Faculty of Science, Taibah University, Almadinah Almonawarah, Kingdom of Saudi Arabia

ABSTRACT

Fusarium wilt, caused by Fusarium oxysporum f.sp. ciceri (Foc), is one of the tremendous biotic stresses instigating huge yield losses in chickpea (Cicer arietinum). A total of 853 microsatellites were screened in two chickpea parents (FLIlP97-7 and ILC482). One parent, FLIP97-7, is Fusarium wilt resistant to the fungus Fusarium oxysporum, while the other parent, ILC482, is susceptible. One hundred forty four recombinant inbred lines, originated from a cross between FLIP97-7 and ILC482, segregating for this character were mapped using SSR markers. The study detected variation between the two parents, where twenty-six Simple sequence repeats (SSRs) were polymorphic. The SSR microsatellites were mapped on the 144 recombinant inbred lines segregating for this character. Linkage analysis showed that the Fusarium vascular wilt resistance locus race 0 (Foc-0) was located on linkage group (1) around 19.7 centi Morgan away from the NCPGR77 microsatellite marker. This study may help in improving the chickpea breeding programs through the development of diagnostic markers that could contribute significantly in Marker Assisted Selection.

Keywords: *Fusarium oxysporum ciceris, Recombinant Inbred Lines (RILs), Molecular markers, SSR, Linkage analysis.*

INTRODUCTION

Chickpea (*Cicer arietinum* L.*)* legume crop is considered globally as the most cultivated grain after dry beans (*Phaseolus vulgaris* L.) (Saxena, 1990). It has a simple short diploid genome size, about 7.3 Gega bases Gb, (2x=2n, n=8 chromosomes). Chickpeas are self-pollinating RabI (spring) crop, sown in winter and harvested in spring (Jain *et al.*, 2013 and Varshney *et al.*, 2013). The total cultivated area is estimated as 13,106 ha producing a total of 13 million tons worldwide (FAOSTAT, 2016). Cultivated chickpea consists of two morphologically deviating types, Desi and Kabuli. The main producing countries for chickpea are India (9,880,000 tones), Australia (629,400 tones) and Pakistan (399,030 tones), representing 67.32%, 6.19% and 5.72%, respectively, of global production. Additionally, in developing countries chickpea is an affordable source of

protein (especially in South Asia) where most of the populations are vegetarians (Gaur *et al.*, 2012). Chickpea improves soil fertility in dry lands through nitrogen fixation.

There is a crucial necessity to develop new cultivars tolerant to various biotic and abiotic limiting factors (Gaur *et al.*, 2012). The soil-borne fungus *Fusarium oxysporum* Schlechtend: Fr. f. sp. *ciceris*, causing Fusarium wilt (FW), is the most critical biotic stress that causes reduction of chickpea yields (Prasad and Padwick). It has been reported that most of the chickpea cultivating areas showed major losses under favorable conditions for the Fusarium wilt (Halila and Strange, 1996 and Nene *et al.*, 1996). It is difficult to control the pathogen, as it is persistent in the soil, even with the nonexistence of the host plant.

Fusarium oxysporum f. sp. *ciceris* (Foc) is recognized by comprising pathogenic variability, i.e. it includes different pathogenic races and pathotypes. Pathogenicity testing can discriminate between the two types of the Fusarium. The disease symptoms of the first type are leaves yellowing, while the other type causes wilting syndrome. There are eight classified physiological races for Foc, i.e. races 0, 1A, 1B/C, 2, 3, 4, 5 and 6. (Sharma and Muehlbauer, 2007, Jiménez-Fernández *et al.*, 2013). The yellowing pathotypes involve races 0 and 1B/C , whereas races 1A , 2 , 3, 4, 5 and 6 belong to the wilting pathotypes (Landa *et al.*, 2006 and Basha *et al.*, 2017).

Although chickpea at the morphological level has a broaddissimilarity, the genetic variation within chickpea genotypes is very narrow and limited (Udupa *et al.*, 1993 and Mantri *et al.*, 2007). Additionally, most of their genetic content are analogous, which consequently leads to difficulties for the breeders to produce new cultivars by classical breeding methods (Mantri *et al.*, 2007). Breeding programs of chickpea could be improved through the modification in manipulating the different molecular genomics tools, for instance the marker assisted selection (MAS) approach, which is the leading approach in crop enhancement (Jones *et al.*, 1997 and Gaur *et al.*, 2012). The efficacy of MAS depends on the association potency between the gene locus controlling the trait of interest and the marker of interest (Singh *et al.*, 2008). For that reason, merging MAS linked to Fusarium wilt into chickpea breeding is a promising tool in improving the efficiency of plant selection (Castro *et al.*, 2013). Developing resistant genotypes against Fusarium wilt is the most effective approach in FW host plant management (Pratap *et al.*, 2017).

Microsatellites are small tandem sequence repeats of DNA, commonly 2–5 bp in length, known as simple sequence repeats (SSRs), which are present in most eukaryotes. They have various applications in the genome mapping and phylogenetic analysis. The precedence of SSRs over other markers is that it has many beneficial features such as co-dominant inheritance, good genome coverage, multiallelic nature, reproducibility, relative abundance and high degree of polymorphism (Powell *et al.*, 1996). Microsatellite markers have been employed with numerous plants for developing genomic maps and tagging different traits of agronomic significance (Winter *et al.*, 1999 and Cho *et al.*, 2000). On the other hand, Inter simple sequence repeat (ISSRs) and Start Codon Targeted polymorphism (SCoTs) markers are random, non-specific targeted markers and are dominant makers that can distinguish the heterozygosity of a genotype. ISSR targets the microsatellite repetitive sequence, while the SCoT targets the genes through the "ATG" opening reading sequence.

In this study, to address the drawback of the limited genetic variability within *C. arietinum*, enrich the genetic map of chickpea,

and improve locating additional interesting genes of agronomic importance, for Fusarium wilt, a map was constructed for a recombinant inbred line (RIL) population segregating for resistance to *Fusarium* with sequence tagged microsatellites (STMS) markers. This would allow performing a qualitative analysis of the Foc-0 region. SSR-PCR reactions were conducted using 853 SSR primers.

MATERIALS AND METHODS

Plant material

Total of 144 F_6 recombinant inbred lines (RILs) of chickpea were developed, bred from a cross between FLIP97-7 (an FW resistant parent) and ILC482 (an FW susceptible parent). This population was obtained from the International Center for Agricultural Research in the Dry Areas (ICARDA**).**

Phenotype evaluation

The average of the FW resistance score for each RIL was obtained from the Legume pathology laboratory at ICARDA. Briefly, the RILs were phenotypically estimated for resistance and susceptibility in a wilt-infected field (called a sick plot) of race 0 of *Foc* at the ICARDA Terbol station (Terbol, Lebanon). Phenotypic data for the 144 recombinant inbred lines were scored as 'R' for a resistant line, 'S' for a susceptible line and '–' for missing data.

DNA extraction, molecular markers and PCR conditions

For DNA extraction, 0.1 grams of fresh young leaf tissue were collected and extraction was performed using the CTAB method according to (Doyle and Doyle 1990). Different types of molecular markers were applied when screening the two parents, seeking any genetic polymorphism that could appear between them, in order to guide us to the Fusarium resistance region; a linkage map was also drawn. PCR reactions were conducted using an 853 simple sequence repeats (SSRs) primers, eighteen Inter simple sequences repeat (ISSRs) and six Start Codon Targeted polymorphism (SCoTs). PCR amplifications were implemented in a total volume of 25 µl of the reaction mixture that comprised, 2.5 mM of $MgCl_2$, 10 mM of dNTPs, 0.2 U of GoTaq® DNA Polymerase (GoTaq_ Flexi, Promega, Madison, Wisconsin, USA). The PCR amplifications were performed in thermal cycler (GeneAmp PCR system 9700, Applied Biosystems)**.** For the SSR primers (Table 1), the cycles were conducted as follows: one cycle of 2 minutes at 95°C, 35 cycles of 30 sec at 95°C, 30 sec at 53-60°C and 72°C for 30 sec, followed by a final extension cycle at 72°C for 7 minutes.

Table (1): Polymorphic SSR primer names, forward and reverse sequences, annealing temperature, motifs and amplified product size.

No.	Primer name		sequence 5'->3'	Ta°C	Amplified size (bp)
1	CaGM20820	F	GGGTTTTGAGGATATGGATGAA	62	300
		R	CAAAACCCTAACCTCTCTCAATC		
2	CaGM20889	F	GAGGCAAATGCGAGAAAATC	54	400
		R	TCAAGACAAATGGGGCCTTA		
3	GA16	F	CACCTCGTACCATGGTTTCTG	55	247
		R	TAAATTTCATCCTCTCCGGC		
4	GA20	F	TATGCACCACACCTCGTACC	55	174
		R	TGACGGAATTCGTGATGTGT		
5	H1K23	F	GACCCTTCTTAATTTGTTTTATGC	55	163
		R	CACTGCTTCCAATGCAATCT		
6	H2I20	F	TGTTTTGCTCATCTGTTAAATCAA	55	190
		R	AGCATGCCTCTGATGAATAGTAAC		
7	H3E052	F	TAGACCCTTGCTTCTTGTTCCT	60	184
		R	AATCTTGTTGGTTCTTTGGTCA		
8	H3H07	F	GAGGCATAGTACCTCAATTTTATTCA	55	289
		R	AAGAAAGACAGGTTATCTGTGTGGT		
9	H5E08	F	GAGAAATTTTATTTGTGGGGATG	55	178
		R	ACTCCCTCATTTTTCTCCTGTTT		
10	H6D11	F	AAAGATGGGAACTTGAGATGTTG	55	200
		R	AATAGCTACTCAAGGCTGAAGAAA		
11	NCPGR50	F	ATGATGGATTTTCGGAATGT	55	209
		R	AAAAATGCTGGAAGGAACTG		
12	NCPGR69	F	GACCGAATGTCCATAAATCA	55	252
		R	GGAGCTGGAAAAACTACAGC		
13	NCPGR74	F	TCCGTCCACACATTTCTACT	55	231
		R	CTTTTAGTTGGTCGAAGCC		
14	NCPGR77	F	TGGACTAACAAATACGACGA	55	225
		R	AGGCCACCCTAAATTTTATT		
15	NCPGR81	F	CCGAATGTCCATAAATCAAT	55	211
		R	TGTTTGACTGGGATAACTCC		
16	NCPGR89	F	AAAGGGCCTTCAAGTTGTAT	55	263
		R	ACTTTTGGAGTGAGAGGCT		
17	TA179	F	CAGAAGACGCAGTTTGAATAACTT	55	218
		R	CGAGAGAGAGAAAGGAAGAAGAG		
18	TA39	F	TTAGCGTGGCTAACTTTATTTGC	55	249
		R	ATAAATATCCAATTCTGGTAGTTGACG		
19	TA42	F	ATATCGAAATAAATAACAACAGGATGG	55	209
		R	TAGTTGATACTTGGATGATAACCAAAA		
20	TA59	F	ATCTAAAGAGAAATCAAAATTGTCGAA	55	250
		R	GCAAATGTGAAGCATGTATAGATAAAG		
21	TA61	F	CCAAAAACATTGACACAACA	55	262
		R	AAGGGGAGATTTGTTAGGTT		
22	TA71	F	CGATTTAACACAAAACACAAA	55	225
		R	CCTATCCATTGTCATCTCGT		
23	TR59	F	AAAAGGAACCTCAAGTGACA	50	174
		R	GAAAATGAGGGAGTGAGATG		
24	TS 71	F	ATTCAACACTCAGTACTACCATTTT	55	220
		R	GATTGTTAAAAGCTTATATCCCTAA		
25	TS43	F	AAGTTTGGTCATAACACACATTCAATA	50	212
		R	TAAATTCACAAACTCAATTTATTGGC		
26	TS53	F	GATCNTTCCAAAAGTTCATTTNTATAAT	55	267
		R	TTAAAGAACTGATACATTCCGATTATTT		

The PCR products were separated in 10% non-denaturing polyacrylamide gels (1X TBE running buffer) and stained with ethidium bromide (Merck, Darmstadt, Germany) (Fig. 2 and 3). For ISSR primers (Table 2), cycles were conducted as follows: one cycle of 2 minutes at 95°C, 40 cycles of 30 sec at 95°C, 1 minute at (40,42,45,46 or 50°C), and 72°C for 2 minutes, followed by a final extension cycle at 72°C for 7 minutes. For SCoT primers (Table 3) cycles were conducted as follows: one cycle of 3 minutes at 95°C, 40 cycles of 1 minute at 95°C, 1 minute at 50°C, and 72°C for 2 minutes, followed by a final extension cycle at 72°C for 5 minutes. The ISSR and SCoT PCR products were separated on 3% agarose gels (Merck, Darmstadt, Germany) and stained by ethidium bromide.

Table (2): ISSR primer names, sequences and required annealing temperatures (Ta°C).

No.	Primer name	sequence (5'-3')	Ta°C
1	ISSR-1	AGAGAGAGAGAGAGAGYC	50
2	ISSR-2	AGAGAGAGAGAGAGAGYG	50
3	ISSR-3	ACACACACACACACACYT	50
4	ISSR-4	ACACACACACACACACYG	50
5	ISSR-5	GTGTGTGTGTGTGTGTYG	50
6	ISSR-6	CGCGATAGATAGATAGATA	50
7	ISSR-7	GACGATAGATAGATAGATA	50
8	ISSR-8	AGACAGACAGACAGACGC	50
9	ISSR-9	GATAGATAGATAGATAGC	50
10	ISSR-10	GACAGACAGACAGACAAT	50
11	ISSR-11	ACACACACACACACACYA	50
12	ISSR-12	ACACACACACACACACYC	50
13	ISSR-13	AGAGAGAGAGAGAGAGYT	50
14	ISSR-14	CTCCTCCTCCTCCTCTT	50
15	ISSR-15	CTCTCTCTCTCTCTCTRG	50
16	ISSR-16	TCTCTCTCTCTCTCTCA	50
17	ISSR-18	HVHCACACACACACACAT	50
18	ISSR-19	HVHTCCTCCTCCTCCTCC	50

Table (3): SCoT primer names and sequences (5'-3'). The ATG sequence is marked in bold in each primer.

No.	Primer name	Sequence (5'-3')
1	SCoT -12	ACGAC**ATG**GCGACCAACG
2	SCoT -14	ACGAC**ATG**GCGACCACGC
3	SCoT -16	ACC**ATG**GCTACCACCGAC
4	SCoT -20	ACC**ATG**GCTACCACCGCG
5	SCoT -22	AACC**ATG**GCTACCACCAC
6	SCoT -28	CC**ATG**GCTACCACCGCCA
7	SCoT -35	C**ATG**GCTACCACCGGCCC
8	SCoT -33	CC**ATG**GCTACCACCGCAG
9	SCoT -36	GCAACA**ATG**GCTACCACC

Band scoring

Gel electrophoresis (both acrylamide and agarose) were documented by a Molecular Imager® Gel Doc™ XR+ System provided with Image Lab™ Software (Bio-Rad™, California, United States). Polymorphisms were scored visually and assembled in an Excel spreadsheet (Excel 2000, Microsoft) using the following codes: 'A' for a homozygous individual with a fragment derived from parent 1 (Flip 97-7); 'B' for a homozygous individual with a fragment derived from parent 2 (ILC 482); 'H' for a heterozygous individual with fragments obtained from both parents; and '–'for missing data. This FW data was converted from 'R' to 'A' and from 'S' to 'B' to integrate the resistant gene with the molecular marker data.

Mapping and linkage analysis

The genotype data was used to develop a genetic linkage map through the computational calculating program, JoinMap v4.0 ® (Van Ooijen, 2006). Markers were grouped at 0.3 as a minimum logarithm of the odds (LOD) score and a maximum recombination fraction of 0.4 as general linkage criteria to create linkage groups. Kosambi's function was used to convert recombination percentages to centi-Morgan map unit distances (Kosambi, 1943). A chi-square test was also calculated by the JoinMap v4.0 ®, to test the deviation from the expected segregation ratio (Van Ooijen, 2006).

RESULTS

The 18 ISSRs and the 6 SCoTs screened in the parents were monomorphic, thus were non informative for the genotyping. Out of 853 SSRs, only 26 (3.0%) were polymorphic between the parents FLIP97-7 and ILC482 (Table 1), and 602 (70.5%) were monomorphic; the remainder were 225 (26.5%) had no amplification products. A total of 144 RILs were genotyped using these polymorphic markers to generate a linkage group(s) and a genetic map was created for the mapping population (Fig. 2 and 3). The ratio of alleles similar to each parent were studied by the chi-square analysis. As indicated in Table (4), the alleles similar to parent 1 denoted (a), and alleles similar to patent 2 denoted (b). The SSR markers that showed significant results were (NCPGR74, H3E052, TA61, ts71,GA16, H6D11 and NCPGR50) in addition to the phenotype marker (foc-0), these markers followed the expected segregating ratio for {allele-(a) : allele-(b)} presenting {1:1}. The other markers showed an elevated X^2 revealing the non-significance The chi-square analysis indicated that 19 (73%) out of 26 microsatellite markers significantly deviated from the 1:1 expected segregating ratio for RIL populations (Table 4).

Table (4): The chi-square analysis of the 26 segregating SSR loci in the RIL population. Chi-square values were estimated using Join Map® 4.0 software. For a: the number of RILs similar to the parent 1 (P1)-type allele. For b: the number of the RILs similar to the parent 2 (P2)-type allele. M: Missing data. *: chi-square value is significant at p<0.05; **: significant at p<0.01; ***: significant at p<0.001; *: significant at p<0.0001; *****, ******, *******: chi-square value is statistically extremely significant.***

Nr	Locus	a	b	missing	X2	Signif.
1	NCPGR74-SSR	67	68	7	0.01	-
2	H3E052-ssr	62	58	4	0.13	-
3	TA61-SSR	63	69	6	0.27	-
4	ts71-SSR	65	56	12	0.67	-
5	GA16-SSR	75	65	4	0.71	-
6	foc-0	75	62	7	1.23	-
7	H6D11-SSR	67	51	19	2.17	-
8	NCPGR50-SSR	78	59	6	2.64	-
9	TS43-SSR	78	54	3	4.36	**
10	TA71-SSR	43	65	31	4.48	**
11	NCPGR77-SSR	75	50	11	5	**
12	TA59-SSR	80	51	10	6.42	**
13	TR59-SSR	80	50	7	6.92	***
14	GA20-SSR	82	51	10	7.23	***
15	CAGM889-SSR	75	45	17	7.5	***
16	caG820-SSR	80	47	9	8.57	****
17	TA179-SSR	84	50	8	8.63	****
18	TA39-SSR	84	49	10	9.21	****
19	TA42-SSR	88	52	3	9.26	****
20	H3H07-SSR	83	48	3	9.35	****
21	NCPGR69-SSR	90	50	4	11.43	*****
22	TS53-SSR	88	48	7	11.76	*****
23	NCPGR89-SSR	85	43	4	13.78	******
24	NCPGR81-SSR	89	44	5	15.23	*******
25	H1K23-SSR	37	103	4	31.11	*******
26	H5E08-SSR	126	18	0	81	*******
27	H2I20-SSR	6	130	4	113.06	*******

Construction of a genetic map

Linkage analysis revealed two linkage groups, LG1 and LG2. A total of 9 markers (H3E052, ts71, NCPGR89, H1K23, H5E08, NCPGR74, TA61, NCPGR50, TA71 and H2I20) were kept unlinked. The first linkage group consisted of 12 markers spanning 37 centiMorgan including TA39, H3H07, caG820, TR59, CAGM889, TS53, TA42, NCPGR69, TA179, TS43, NCPGR81, NCPGR77, with an average marker distance of 3 cM between the markers. The second LG2 consisted of 4 markers (GA20, GA16, H6D11 and TA59) spanning 51 cM with an average of 12.75 cM between the markers (Fig. 1).

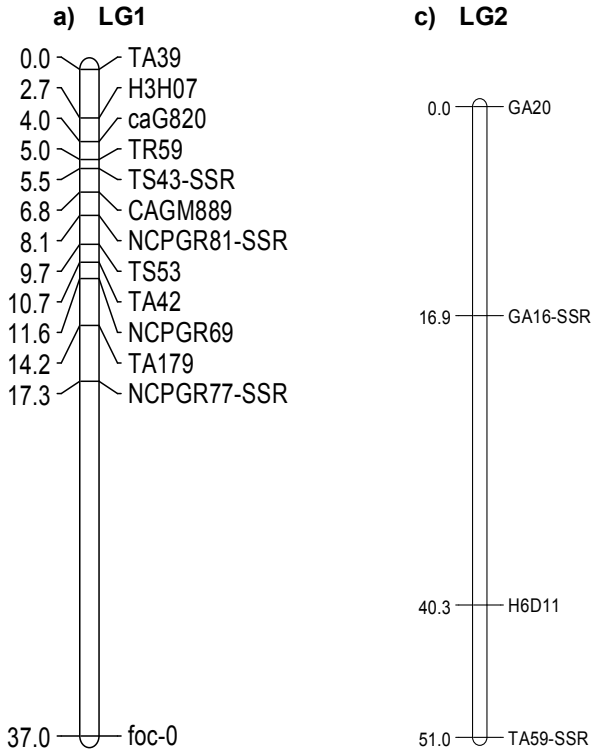

Fig. (1): A genetic linkage map of Cicer sp. based on microsatellites and the foc-0 marker. The map shows the position of microsatellites at LOD score ≥ 3. The values on left side of the individual linkage groups represents centi morgan calculated using kosambi mapping function. a): LG1: linkage group 1, b): LG2: linkage group 2.

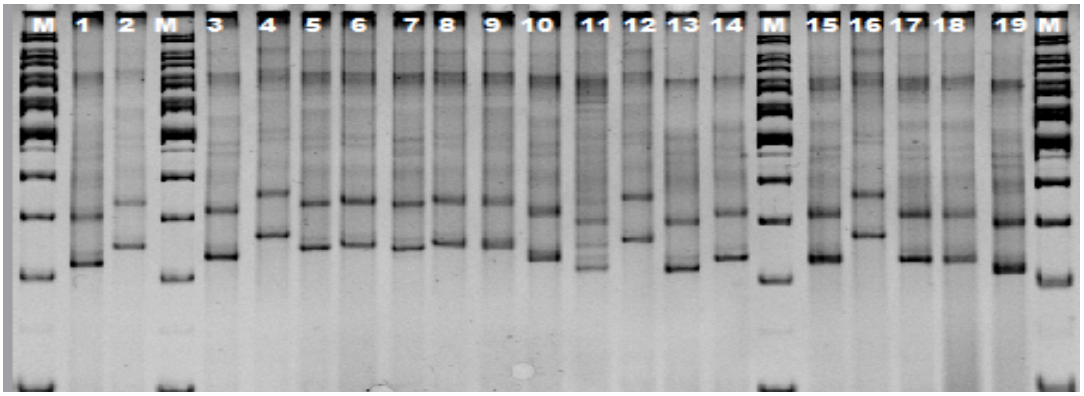

Fig. (2): Segregation of Ts43 SSR microsatellite in the RIL population. The PCR products were electrophoresed on a 10% polyacrylamide gel and stained with ethidium bromide. Lane 1: Flip97-7 (P1), lane 2: ILC482 (P2), and lanes 3-19 RILs. M refers to the DNA ladder 100bp.

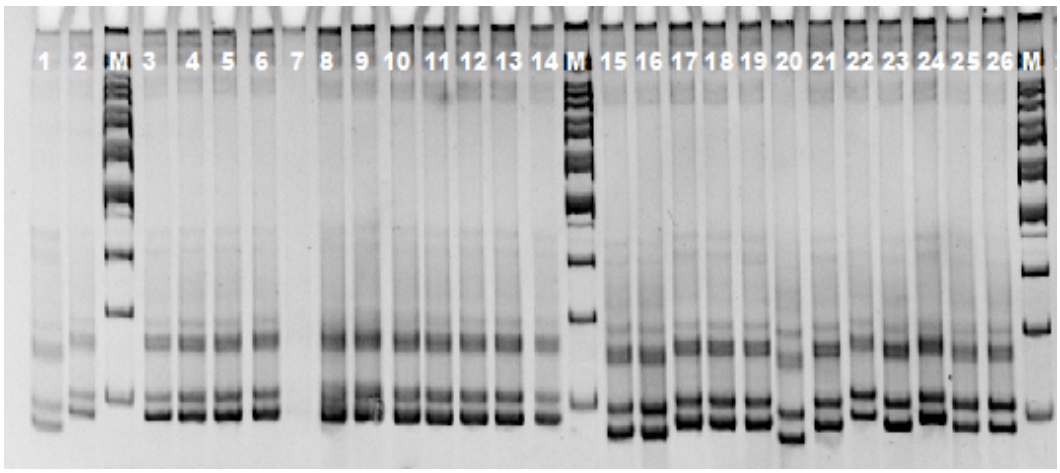

Fig. (3): Segregation of Ts43 SSR microsatellite in the RIL population. The PCR products were electrophoresed on a 10% polyacrylamide gel and stained with ethidium bromide. Lane 1: Flip97-7 (P1), lane 2: ILC482 (P2), and lanes 3-19 RILs. M refers to the DNA ladder 100bp.

Identification of the Fusarium vascular wilt resistance locus

The population segregated for 75 resistant versus 62 susceptible RILs, which is in accordance with a 1:1 segregation ratio (x^2=1.23; df=1; p>0.05), pointing out that the Fusarium vascular wilt resistance is controlled by only one gene (Fw). Our linkage analysis revealed that the Fusarium vascular wilt resistance locus (Foc-0) marker was localized on LG1 around 19.7 cM away from the NCPGR77 marker.

DISCUSSION

Minor genetic variation is present in *Cicer arietinum*. Thus, it is difficult for breeding efforts to develop new varieties resistant to stress conditions through classical breeding methods (Mantri *et al.* 2007). A study by Chowdhury *et al.*(2002), revealed that an elevated similarity within chickpea breeding lines, in addition to different varieties, were verified to be genetically analogous. Another study determined that cultured chickpea genotypes had high morphological differences, however, genetic variation was minimal (Mantri *et al.*, 2007). ISSR and SCoT markers are dominant markers which cannot discriminate between homozygote and heterozygote genotypes. In addition they have demerit of being random, non-specific targeted markers. SCoT marker is gaining popularity for its superiority over other dominant DNA marker systems like RAPD and ISSR for higher polymorphism and better marker resolvability. The SCoT primers are based on conserved regions flanking the initiation codon sequences of genes. It shares the principle of using a single primer like RAPD and ISSR (Ahmad and Talebi, 2017 and Maisuria *et al.*, 2017). The ISSR marker targets interspecificaly the microsatellite repetitive sequence, while the SCoT targets the genes through the "ATG" start codon opening reading sequence through all the genome. The purpose of using the ISSR and SCoT was look forward to find any genetic variation in between the two parents of chickpea, especially the SCoT, as it targets genes, if there were any difference between the 2 parents this may have guided us to a gene found in one parent and absent in the other which may have conclude the genetic region responsible for the resistant response of the plant. Monomorphism is the state where all the individuals have the same form (genotype), while polymorphism is a marker or band that present in an individual but absent in another. In our study, all the 6 SCoT and 18 ISSR markers were monomorphic, thus were non informative for the genotyping (Iruela *et al.*, 2002). In addition, most of the SSR microsatellite markers were also monomorphic, although, the parents (FLIP97-7 and ILC482) showed phenotypic differences with regard to Fusarium wilt resistance. In a previous study, high rate of monomorphism was observed, in which 38% of the primers were found to be polymorphic, 26% were monomorphic and 36% did not show any amplification (Ratnaparkhe *et al.*, 1998). Another study, observed that 48 (23%) out of 201 SSR markers were polymorphic and 153 (76%) were monomorphic (Jingade *et al.*, 2014). Low polymorphism results were also reported by (Benko-Iseppon *et al.*, 2003), where only 24 (5.5%) out of 432 markers were polymorphic and 242 were monomorphic; the rest which contributes 166 did not show any amplification product.

SSR or microsatellite markers, when compared against other marker types such as isozymes, RFLPs, RAPDs and AFLPs (Muehlbauer *et al.*, 1989, Udupa *et al.*, 1993 and Eujayl *et al.*, 1998), proved to be more

valuable in overcoming the problem of low polymorphism (Lichtenzveig *et al.*, 2005).

This superiority of SSR is due to the co-dominance character and specificity of the marker nature itself, in contrast with the other markers. A previous study stated that with regard to the results of the difference index, polymorphic information content (PIC), as a value of a marker for detecting polymorphism within a population and similarity probabilities, SSR marker was effective in the management of chickpea, barley and soybean genetic sources (Kraic *et al.*, 2002 and Samyuktha *et al.*, 2018). The purpose of this study was to locate the FOC-0 region in the chickpea. We performed a qualitative analysis for the Foc-0 region and our linkage analysis revealed that the Fusarium vascular wilt resistance locus (Foc-0) marker was localized on LG1, approximately 19.7 cM away from the NCPGR77 marker. There is a distinct similarity between our map and that of other studies (Millan *et al.*, 2010, and Jendoubi *et al.*, 2016) with respect to the genetic locations. The markers (TS43, Ta42, Ta39, CaGM20889, CaGM20820) on LG1 were found on chromosome 5 (Ca5) and are comparable with the results from the study performed by (Jendoubi *et al.*, 2016). The Fusarium vascular wilt resistance was localized to chromosome 5 and this resistance gene was flanked by microsatellite markers CaGM20820 and CaGM20889. The distance between CaGM20820 and CaGM20889 on Ca5 is estimated to be around 2 cM as a genetic distance, covering about 740 Mb as a physical distance (16759541–17501349 bp) (Jendoubi *et al.*, 2016, and Pratap *et al.*, 2017).

The chi-square analysis indicated that 19 (73%) out of 26 microsatellite markers significantly deviated from the expected 1:1 ratio, which can explain that the Fusarium vascular wilt resistance is controlled by only one gene (Fw). This high segregation distortion may be related to natural selection (natural wilt infection) through several generations during population development. This may explain the high deviation towards the resistant genotype in most of the microsatellite markers as shown in Table 4.

This high segregation distortion was also observed in a previous study of the chickpea, where a chi-square analysis showed that 10 out of 19 loci deviated significantly ($p = 0.05$) from the expected Mendelian segregation ratio (Benko-Iseppon *et al.*, 2003). In another study, the chi square test identified more than 24% of the markers used in that study did not segregate in agreement with the expected ratio of Mendelian inheritance (Jamalabadi *et al.*, 2013). As reported above, our linkage analysis revealed that the Fusarium vascular wilt resistance locus (Foc-0) marker was localized on LG1, approximately 19.7 cM away from the NCPGR77 marker. In conclusion our study would aid in the development of diagnostic markers that could be a promising tool for Marker Assisted Selection in the upcoming chickpea breeding programs.

Compliance with Ethical Standards

Conflict of interest

The authors declare that they have no conflict of interest.

Human studies and participants

This article does **not contain** any studies with human participants or animals performed by any of the authors.

Informed consent

Informed consent was obtained from all individual participants included in the study.

ACKNOWLEDGMENTS

This research was financially supported by the International Center for Agricultural Research in the Dry Areas, (ICARDA). In addition to, the Grains Research and Development Corporation (GRDC), Australia. The entire laboratory facilities were provided by the Agricultural Genetic Engineering Research Institute, (AGERI), Egypt.

REFERENCES

Ahmad, K. M.-S. and Talebi, R. (2017). Genetic diversity assessment in chickpea using morphological characters and start codon targeted (SCoT) molecular markers. Annu Res Rev Bio. 15(1).

Basha, S. J.; Sarma, A. S. R. and Ahammed, S. K. (2017). Agronomic manipulations for pests and diseases management in Chickpea: A Review. Int J Pure Appl Biosci. 5(2), 842-849.

Benko-Iseppon, A. M.; Winter, P. Huettel, B.; Staginnus, C.; Muehlbauer, F. and Kahl, G. (2003). Molecular markers closely linked to fusarium resistance genes in chickpea show significant alignments to pathogenesis-related genes located on Arabidopsis chromosomes 1 and 5. Theor Appl Genet. 107(2), 379-386.

Castro, P.; Rubio, J. and Madrid, E. (2013). Efficiency of marker-assisted selection for ascochyta blight in chickpea. J Agricult Sci. 1-12.

Cho, Y. G.;Ishii, T.; Temnykh, S.; Chen, X.; Lipovich, L.; McCouch, S. R.;Park, W. D.; Ayres, N. and Cartinhour, S. (2000). Diversity of microsatellites derived from genomic libraries and GenBank sequences in rice (Oryza sativa L.). Theor Appl Genet. 100(5), 713-722.

Doyle, J. J. and Doyle , J. L. (1990). Isolation of plant DNA from fresh tissue. Focus 12, 13-15.

Eujayl, I.; Baum, M.; Powell, W.; Erskine, W. and Pehu, E. (1998). A genetic linkage map of lentil (Lens sp.) based on RAPD and AFLP markers using recombinant inbred lines. Theor Appl Genet. 97(1), 83-89.

FAOSTAT. (2016). http://faostat3.fao.org.

Gaur, P. M.; Jukanti, A. K.;and Varshney, R. K. (2012). Impact of Genomic Technologies on Chickpea Breeding Strategies. Agron J. 2(3), 199.

Halila, M. and Strange, R. (1996). Identification of the causal agent of wilt of chickpea in Tunisia as Fusarium oxysporum f. sp. ciceri race 0. Phytopathologia mediterranea. 67-74.

Iruela, M.; Rubio, J.; Cubero, J.;Gil, J. and Millan, T. (2002). Phylogenetic analysis in the genus Cicer and cultivated chickpea using RAPD and ISSR markers. Theor Appl Genet. 104(4), 643-651.

Jain, M.; Misra, G.; Patel, R. K.; Priya, P.; Jhanwar, S.; Khan, A. W.; Shah, N.; Singh, V. K.; Garg, R. and Jeena, G. (2013). A draft genome sequence of the pulse crop chickpea (Cicer arietinum L.). Plant J. 74(5), 715-729.

Jamalabadi, J. G.;Saidi, A.; Karami, E.; Kharkesh, M. and Talebi, R. (2013). Molecular mapping and characterization of genes governing time to flowering, seed weight, and plant height in an intraspecific genetic linkage map of chickpea (Cicer arietinum). Biochem genet. 51(5-6), 387-397.

Jendoubi, W.;Bouhadida, M.; Millan, T.; Kharrat, M.; Gil, J.; Rubio, J. and Madrid, E. (2016). Identification of the target region including the Foc0. Euphytica. 210(1), 119-133.

Jiménez-Fernández, D.;Landa, B. B.; Kang, S.; Jiménez-Díaz, R. M. and Navas-

Cortés, J. A. (2013). Quantitative and microscopic assessment of compatible and incompatible interactions between chickpea cultivars and Fusarium oxysporum f. sp. ciceris races. PloS one. 8(4), e61360.

Jingade, P.; Bhosale, L. V.; Sanjayrao, J. A.; Rajanna, R.; Jain, M. and Ravikumar, R. L. (2014). Characterization of microsatellite markers, their transferability to orphan legumes and use in determination of genetic diversity among chickpea (Cicer arietinum L.) cultivars. J Crop Science and Biotech. 17(3), 191-199.

Jones, N.;Ougham, H. and Thomas, H. (1997). Markers and mapping: we are all geneticists now. The New Phytologist. 137(1), 165-177.

Kosambi, D. D. (1943). The estimation of map distances from recombination values. Annals of Eugenics. 12(1), 172-175.

Kraic, J.; Gregova, E.; Jomova, K. and Hudcovicova, M. (2002). Microsatellite markers discriminating accessions within collections of plant genetic resources. Cell Mol Biol Lett. 7(2B), 745-752.

Landa, B. B.; Navas-Cortés, J. A.; del Mar Jimenez-Gasco, M.; Katan, J.; Retig, B. and Jiménez-Díaz, R. M. (2006). Temperature response of chickpea cultivars to races of Fusarium oxysporum f. sp. ciceris, causal agent of Fusarium wilt. Plant dis. 90(3), 365-374.

Lichtenzveig, J.; Scheuring, C.; Dodge, J.; Abbo, S. and Zhang, H.-B. (2005). Construction of BAC and BIBAC libraries and their applications for generation of SSR markers for genome analysis of chickpea, Cicer arietinum L. Theor Appl Genet. 110(3), 492-510.

Maisuria, H. J., Patel, R. M., and Suthar, K. P. (2017). Validation of Molecular Markers Linked to Fusarium Wilt Resistance in Chickpea Genotypes. Int J Pure Appl Biosci. 5(1), 254-260.

Mantri, N. L.; Ford, R.; Coram, T. E. and Pang, E. C. (2007). Transcriptional profiling of chickpea genes differentially regulated in response to high-salinity, cold and drought. BMC Genomics. 8(1), 303.

Millan, T.; Winter, P.; Jüngling, R.; Gil, J.; Rubio, J.; Cho, S.; Cobos, M.; Iruela, M.; Rajesh, P. and Tekeoglu, M. (2010). A consensus genetic map of chickpea (Cicer arietinum L.) based on 10 mapping populations. Euphytica. 175(2), 175-189.

Muehlbauer, F.; Weeden, N. and Hoffman, D. (1989). Inheritance and linkage relationships of morphological and isozyme loci in lentil (Lens Miller). J Heredity. 80(4), 298-303.

Nene, Y.; Sheila, V. and Sharma, S. (1996). A world list of chickpea and pigeonpea pathogens. Patancheru. 502, 324.

Powell, W.; Machray, G. C. and Provan, J. (1996). Polymorphism revealed by simple sequence repeats. Trends plant sci. 1(7), 215-222.

Prasad, N. a. and Padwick, G. W. (1939). The genus Fusarium 11. A species of Fusarium as a cause of wilt of gram (C. arietinum L.). Indian Agri. Sci. 9: 731.

Pratap, A.; Chaturvedi, S. K. ; Tomar, R.; Rajan, N. ; Malviya, N.; Thudi, M.; Saabale, P.; Prajapati, U.; Varshney, R. K. and Singh, N. (2017). Marker-assisted introgression of resistance to fusarium wilt race 2 in Pusa 256, an elite cultivar of desi chickpea. Mol Gen Genomics. 292(6), 1237-1245.

Ratnaparkhe, M.; Santra, D.; Tullu, A. and Muehlbauer, F. (1998). Inheritance of inter-simple-sequence-repeat polymorphisms and linkage with a fusarium wilt resistance gene in chickpea. Theor Appl Genet. 96(3), 348-353.

Samyuktha, S.; Bapu, J. K. and Geethanjali, S. (2018). Molecular Genetic Diversity and Population Structure Analysis

in Chickpea (Cicer arietinum L.) Germplasm using SSR Markers. Int. J. Curr. Microbiol. App. Sci. 7(2), 639-651.

Saxena, M. C. (1990). Problems and potential of chickpea production in the nineties. Pages 13-2 in: Chickpea in the Nineties: Proc. Int. Workshop Chickpea Improvement, 2nd. International Crop Research Institute for Semi-Arid Tropics, Andhra Pradesh, India.

Sharma, K. D. and Muehlbauer, F. J. (2007). Fusarium wilt of chickpea: physiological specialization, genetics of resistance and resistance gene tagging. Euphytica. 157(1), 1-14.

Singh, R.; Sharma, P.; Varshney, R. K.; Sharma, S. and Singh, N. (2008). Chickpea improvement: role of wild species and genetic markers. Biotechnol Genet Eng Rev. 25(1), 267-314.

Udupa, S.; Sharma, A.; Sharma, R. and Pai, R. (1993). Narrow genetic variability in *Cicer arietinum* L. as revealed by RFLP analysis. J Plant Biochem Biotechnol. 2(2), 83-86.

Van Ooijen, J. (2006). JoinMap 4: Software for the calculation of genetic linkage maps in experimental populations. Kyazma BV, Wageningen, the Netherlands.

Varshney, R. K.; Song, C.; Saxena, R. K.; Azam, S.; Yu, S.; Sharpe, A. G.; Cannon, S.; Baek, J.; Rosen, B. D. and Tar'an, B. (2013). Draft genome sequence of chickpea (*Cicer arietinum*) provides a resource for trait improvement. Nature biotechnol. 31(3), 240-246.

Winter, P.; Pfaff, T.; Udupa, S. M.; Hüttel, B.; Sharma, P. C.; Sahi, S.; Arreguin-Espinoza, R.; Weigand, F.; Muehlbauer, F. J. and Kahl, G. (1999). Characterization and mapping of sequence-tagged microsatellite sites in the chickpea (Cicer arietinum L.) genome. Molecul General Genet, 262(1): 90-101.

الملخص العربي

استحداث خريطة ارتباط وراثية للحمص *Cicer arietinum* إستناداً إلى الواسمات الجزيئية وتحديد الموضع الجيني لمقاومة ذبول الفيوزاريوم الوعائي

نورهان فؤاد[١]،علاء الدين حموية[١]، أمينة عبد الحميد[٢]، محمد عيد سعد[٢،٤]، محمد محمد يوسف[٣]

[١]التنوع البيولوجي والبرنامج المتكامل لإدارةالجينات،المركزالدولي للبحوث الزراعية في المناطق الجافة، الجيزة، مصر.

[٢]قسم الحمض النووي والبروتيوميات وعلم الجينوم،مختبرمركزالجينوميات،معهدبحوث الهندسة الوراثية الزراعية ،مركزالبحوث الزراعية ،الجيزة ،مصر.

[٣]قسم الكيمياء، كليةالعلوم، جامعة القاهرة، الجيزة، مصر.

[٤]قسم الأحياء، كلية العلوم، جامعة طيبة، المدينة المنورة، المملكة العربية السعودية.

يعتبرنبات الحمص واحدا من أهم انواع البقوليات في البلدان النامية ، كما يعتبر مصدر نباتي غني بالبروتين، الذي يلعب دورا حيويا في الأمن الغذائي في تلك البلدان. كما يلعب دورا هاما كعلف لتغذية الحيوان في نظم الثروة الحيوانية. تعتبر انتاجية الحمص في مصر من أعلى الإنتاجيات في العالم، حيث تعادل انتاجية مصر حوالي ضعف انتاجية الهند على مستوى الهكتار الواحد، و التي تقدر بحوالي ٢ طن للهكتار، بينما الهند أقل من الطن للهكتار (حوالي٠،٩طن للهكتار) . بالرغم من ذلك، فإن الأخيرة تعتبر هي الدولة الأولى عالميا في إنتاج محصول الحمص، حيث تنتج حوالي ٧مليون طن (حسب إحصائيات منظمة الفاو ستات لعام ٢٠١٦). بينما يصل الانتاج في مصر الى ٣ الاف طن فقط و يرجع ذلك إلى عدم اهتمام مصر بالشكل الكافي بزراعة هذا المحصول الهام اقتصاديا. تتلخص أهداف هذه الدراسة في تطوير خريطة ارتباط وراثية للحمص، باستخدام واسمات Microsatellites--SSR ، و محاولة فك شفرة الجينوم؛ وفحص و اختيار الأشكال الظاهرية لنبات الحمص المتعلقة بالمقاومة لمرض ذبول الفيوزاريم ودراسة نسبة الانعزال للجينات المقاومة، و الانتخاب بواسطة الواسمات الجزيئية، و اخيراً تحديد الجينات المتوقع ارتباطها بمرض الذبول في الحمص و استخدامها في الإنتخاب . يعتبر مرض ذبول الفيوزاريوم ، الناجم عن (Foc) *Fusarium oxysporum*f.sp. ciceri ، هو واحد من أهم المخاطر الحيوية التي تصيب النبات و التي بدورها تؤدي إلى خسائر كبيرة في إنتاج محصول الحمص. باستخدام أحد أنواع الواسمات الجزيئية و التي تساهم في الكشف عن مدى ارتباط الصفات الوراثية بالتركيب الجيني ، تم فحص عدد ٨٥٣ واسم جزيئي SSR-Microsatellites في أباءعشيرة الحمص المنتقاه. تتكون هذة العشيرة من الأب الأول (FLIP97-7) وهوالصنف المقاوم لمرض ذبول الفيوزاريوم ،في حين أن الأب الثاني(ILC482) هوالصنف الحساس و القابل للأصابة، بالأضافة إلي مائة وأربعة وأربعين سلالة recombinant inbred lines ،و التي نشأت من تزاوج الصنفين7-FLIP97 و ILC482. وقد كشفت هذه الدراسة عن تباين بين الأبين في ستة وعشرون واسم(SSRs) و من ثم تم تطبيق هذه الواسمات على السلالات(الأبناء). وأظهر تحليل الارتباط أن الموقع الجيني المرتبط بمقاومة مرض ذبول الفيوزاريوم يقع على مجموعة الارتباط (١) على مسافة بعد وراثي ١٩،٧ centi Morgan بعيدًا عن الواسم الجزئي(SSR-NCPGR77). هذه الدراسة سوف تساعد في التغلب على مشكلة إنخفاض التباين الوراثي في محصول الحمص، إضافة إلى تحسين الخريطة الوراثية الخاصة بالحمص وتحديد الجينات الهامة للزراعة.علاوة على المساهمة في تحسين برامج تربية الحمص من خلال تطوير واسمات تشخيصية مهمة لأنتخاب صفات مرغوبة في الحمص.