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Original Research

Molecular and agro- morphological genetic diversity assessment of Chickpea mutants induced via ethyl methane sulfonate

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Abstract: Iran, especially its western provinces, is one of the most chickpea producing countries of the world with the yield about 500 kg/ ha in average. Narrow genetic variability for chickpea is one of the most limitations in conventional breeding approaches. In this study, derived genetic variation among 94 chickpea (Bivanij cultivar) mutant lines produced by Ethyl Methane Sulfonate (EMS) were assessed based on ISSR, RAPD markers in M4 and morpho-agronomic traits in M3 generation. The induced variation via EMS in field experiment, showed significant differences among mutant lines based on almost measured traits. In overall, banding patterns of 6 ISSR primers and 8 RAPD primers revealed 21 (50%) and 24 (42.25%) polymorphic bands, respectively. The ranges of similarity coefficient in ISSR and RAPD markers were 0.62-1.00 and 0.72-1.00, respectively. Specific grouping was carried out by each cluster analysis including ISSR, RAPD, ISSR+RAPD and morpho-agronomic markers based on their similarity matrices. The results showed significant variation generated by EMS based on molecular markers and morpho-agronomic traits. Mantel tests between extracted similarity matrices from each marker system were statistically significant. It could be concluded that the generated variation with EMS as a chemical mutant can be used for chickpea breeding purposes.

Key words: Chickpea, EMS, Mutant, ISSR, RAPD.

Introduction

The world population is predicted to increase to billion by middle of current century, so will require 70–110% rise in food production. Nowadays, greatest challenge facing scientist is ensuring global food security and environment protection (1). Chickpea (*Cicer arietinum* L.) belongs to Fabaceae family as a one of the most important pulse crop is major source of protein for millions of people of the world especially in developing countries (2). With respect to production and area under cultivation and based on FAO states, Iran is one of the major chickpea producer countries of the world. In this country, chickpea is the most important pulse crop. Chickpea is cultivated in Iran about 500000ha of which 95% are grown under rained conditions especially in western provinces. Average chickpea yield in Iran is about 400 to 600 kg ha, which is well below the world average of 900 kg (3).

Narrow genetic variability for chickpea is one of the most breeding challenges in conventional approaches to plant breeding. To overcoming this limit, mutation breeding is used as an important tool to improvement of economically desire traits and/or eliminating undesirable traits through the induction of mutations (4). Successfully induction of genetic variability for several important traits through mutations has been demonstrated by several experiments such as, yield parameters (5, 6, 7-8), the induction of chlorophyll mutations (9, 10-11), early flowering (12), male sterility (13), herbicide tolerance (14) and morphological mutations (15- 16).

Various approaches have been developed for mutagenesis involving chemical, irradiation, and insertional methods. Alkylating agents such as ethyl methane sulfonate (EMS), as a chemical mutagenic compound, induce modification of nucleotides, which results in mispairing and base changes (17).

Different kinds of mutagenesis agents (especially EMS and Gamma rays) with various levels have used for inducing variation in chickpea for the different desire traits such as earliness, yield, blight resistant, wilt tolerant, suitable for saline soils, suitable for irrigated, drought resistance and wilt resistant (18).

In this research, genetic diversity among some chickpea (Bivanij cultivar) mutants produced by Ethyl Methane Sulfonate were analyzed based on ISSR, RAPD markers in M4 and morpho-agronomic traits in M3 generation.

Materials and Methods

Healthy seeds of chickpea) *Cicer arietinum* L.) var. Bivanij were treated (200 seeds for each treatment) with different concentrations of EMS (0.1% and 0.5%) for 8, 16 and 24 hours. The M0 generation was cultivated in March 11th, 2013, with 30 and 5 cm as row spacing and distance between plants, respectively. Seeds of survived plants were cultivated in separates lines as M2 and M3 generations in next years (M2 and M3 in March 9th 2014 and March 24th 2015, respectively) without any selection as Augment design in four blocks. Five plants were randomly harvested in M3 generation and

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global traits of chickpea were measured, phonological traits included. Field and laboratory experiments were performed in research field and Biotechnology Laboratory of Campus of Agriculture and Natural Resources, Razi University of Kermanshah, Iran, respectively. Young fresh leaves were harvested from M4 germinated seeds for DNA isolation and molecular analysis. DNA extraction carried out based on CTAB method described by Murray and Thompson, (19). Finally, DNA samples were stored in -20 °C before ISSR and RAPD analyses. In RAPD and ISSR amplification, six ISSR and eight RAPD markers used for screening and exhibiting genetic variation among all mutants (Table 3). PCR reaction was performed in a total volume of 25 µl in a FLEXCYCLER thermocycler. The reaction mixture including 2.5 µl PCR buffer (10 mMTris-HCL, 50 mM KCL), 1.6 µl MgCl2 (10 mM), 2.5 µl primer (10µM), 0.4 dNTP mix (0.1mM), 2.5 µl template DNA (5 ng/µl), 0.2 µl *Taq* polymerase (5U) and 15.3 µl DDW. Other steps conducted according to Williams et al. (20). After PCR operation, amplified products were run in 1.2% agarose gel with 0.5×TBE and 1 Kbp DNA ladder. After that, gels were stained with ethidium bromide and visualized via ultra violet. Statistical analysis Quantitative analyses of morpho-agronomic traits carried out using SAS (21) software (analysis of variance and comparison of means with LSD test) and SPSS 16.0 (cluster analysis based on Euclidean distance square). In order to molecular analysis, all amplified bands for each marker among all accessions were scored for the absence (0) or presence (1). MVSP software version 3-13r and NTSYS-pc software version 2.02 were used for cluster analysis, performed via Centroid method and Principle coordinate analysis (PCoA), respectively. Finally, the Mantel`s test (22) was performed via XLSTAT software. Mutagenesis effectiveness was calculated on the basis of formula suggested by Froese-Gertzen *et al* (23).

$$\text{Mutagenesis effectiveness} = \frac{Mutation\ rate}{[Conc.of\ mutation\ in\ percent] * [Time\ of\ treatment\ in\ h]}$$

Results and Discussion

Mutagenesis effectiveness

Mutagenesis effectiveness was calculated for each treatments showing in Table1. Among EMS treatments, effectiveness decreased with increase in concentration of EMS as well as time of treatment. Decrease in mutagenesis effectiveness at higher concentration of EMS (0.4%) was observed in Chickpea mutants in Wani and Anis (24) research.

Morpho-agronomical analysis

The results of induction of variation via EMS in field experiment showed significant differences among mutant lines based on almost measured traits. Mean, maximum, minimum and LSD values for each trait and coefficient of variation (CV) values across all 94 mutant lines plus control (Bivanij without mutation treatment) are shown in Table 2 except two traits including harvest index (HI) and hundred seed weight (HSW). The Bivanij (control as not treated by EMS) showed excellence in mostly traits including yield (5.14), height (30.57) and number of seeds per plant (17.87), In contrast, mutant No. 92 had lowest values of same traits. Earlier maturated mutant line was 41 by 94 days to maturation and mutant No. 54 showed opposite status (106 days to maturation). The mean performance of different quantitative traits in a research conducted by Wani and Anis (24) was significantly better among the mutant lines as compared to the control. Cluster analysis based on measured traits classified accessions into separate groups (Fig 1).

Table1. Mutagenesis effectiveness of EMS treatment in Chickpea) *Cicer arietinum L.*) var. Bivanij.

Treatments		Number of treated seeds	Number of survival plants	Mutagenesis effectiveness
0.1 %	8 hours	200	28	0.175
	16 hours	200	13	0.062
	24 hours	200	20	0.025
0.5 %	8 hours	200	13	0.016
	16 hours	200	12	0.016
	24 hours	200	9	0.003

Table 2. The morphological characters and basic statistical data of Chickpea mutants.

Trait	mean	max	min	LSD (5%)	CV (%)	P*(F-test)
Yield (gr)	3.77	5.14	2.72	0.023	2.54	<.0001
Plant Height (cm)	23.70	30.57	16.67	0.119	2.12	<.0001
Number of pods per plant	12.27	16.52	6.73	0.183	6.40	<.0069
Number of seeds per pod	1.06	1.24	1.00	0.006	2.63	<.0253
Number of seeds per plant	13.03	17.87	7.05	0.204	6.70	<.0211
Biological yield	12.70	19.49	7.86	0.165	5.49	<.0016
Harvest Index	0.30	0.35	0.28	0.011	5.08	<.7209
Number of branches	8.03	10.25	3.50	0.106	5.06	<.0094
Hundred seed weight (gr)	29.15	39.06	25.36	0.640	9.15	<.4451
Days to germination	5.02	7	4	0.011	0.87	<.0001
Days to 50% flowering	52.77	56	48	0.040	0.32	<.0001
Days to 50% podding	64.74	69	57	0.011	0.06	<.0001
Days to maturity	101.23	106	94	0.012	0.04	<.0001

p < 0.001= highly significant.

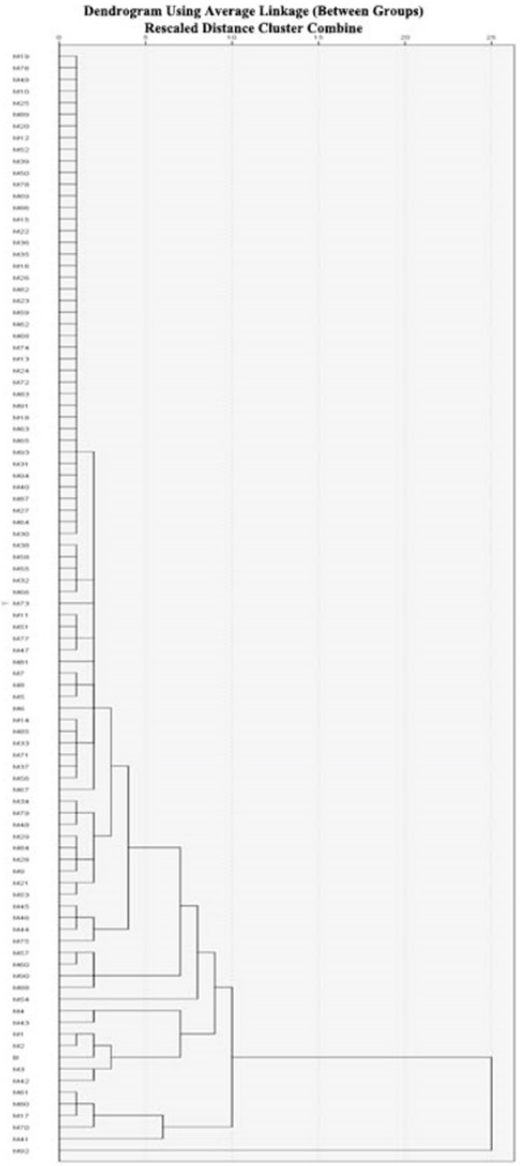

Figure 1. Dendrogram derived from cluster analysis based on morpho-agronomic traits of Chickpea mutants. B= Bivanij (control as not treated by EMS).

DNA molecular marker analysis

Among the 10 ISSR primers in molecular section, only 6 of them successfully amplified polymorphism bands. The all ISSR primers amplified 21 polymorph bands out of 42 (50%). The number of bands varied from three (primer UBC-855) to 10 (primer UBC-857). The highest and lowest percentages of polymorphism belonged to UBC-856 primer (67%) and UBC- 855 primer (40%), respectively. The 8 RAPD (out of 12 used primers) primers that used in this study, produced 57 bands which 24 of them were found to be polymorphic (42.25%), varied from 3 (primer AB1) to 10 (primers OPC08 and C16). Sandhu et al (25) found 13 suitable RAPD primers with high polymorphism to discriminate rice mutant lines produced via physical and chemical mutagenesis EMS included. The PIC index for ISSR primes ranged from 0.09 to 0.50 and for RAPD were from 0.15 from 0.50 (table 3). The PIC value has been used for evaluate genetic variation in many studies (26).

Molecular markers analysis

Cluster analysis based on Jaccard similarity matrix via UPGMA method were performed for ISSR, RAPD and ISSR+RAPD binary data in order to understand genetic relationships among mutants. Molecular markers are a useful complementary tool for morphological and physiological characterization of plants; because they have many advantages for example they are plentiful, independent of environmental effects and cultivar identification early in plant development (27). According to the Jaccard's similarity matrix for ISSR data, the amount of similarity varied from 0.62 (between line 90 and 94) to 1.00 (between some of lines for example 2 and 3) with 0.91 in average (data not shown). The dendrograms are illustrated based on UPGMA analysis of the ISSR, RAPD and RAPD+ISSR data in Figure 2. Based on Jaccard similarity matrix for RAPD data, the most similar lines (full similarity) observed between some of them for example 1 and 3 while, the least of it (0.72) observed among lines 81 and 94. The average of similarity (0.93) was just a little more than what found in ISSR primers (0.91). The main reason for the difference in resolution of RAPD and ISSR is that the two marker techniques targeted different parts of the genome (28). When all binary data from two molecular marker systems were gathered, the resulted dendrogram was more similar to ISSR cluster rather than RAPD one, which might be due to higher number of ISSR primers in comparison to RAPD primers.

Principle coordinate analysis

The principle coordinate analysis was performed with ISSR, RAPD and ISSR+RAPD data in order to establish the relationship among samples and comparison to cluster analysis (Fig 3). Distribution pattern of accessions in this aspect was mainly similar to the result extracted from cluster analysis. The Mantel test also could represent a significant correlation between RAPD and ISSR markers based on their similarity matrices in the level of 0.05.

Table 3. ISSR and RAPD primers and their amplification results in chickpea mutants.

No.	Primer	Primer's sequence	PB	TNB	PIC	No.	primer	Primer's sequence	PB	TNB	PIC
ISSR		3`→5`	(%)			RAPD		3`→5`	(%)		
1	UBC-857	(AC)8T	50	10	0.37	1	OPC08	TGGACCGGTG	70	10	0.30
2	UBC-856	(ACAC)4YG	67	9	0.15	2	OPC15	GACGGATCAG	63	8	0.39
3	UBC-855	(AC)8YT	40	8	0.45	3	U11	AGACCCAGAG	13	8	0.50
4	UBC-818	(CA)8G	43	3	0.45	4	AB1	CCGTCGGTAG	33	3	0.45
5	UBC-873	(ATG)6	50	7	0.49	5	OPC13	AAGCCTCGTC	67	9	0.15
6	UBC-864	(ATG)4	50	5	0.42	6	C16	CACACTCCAG	20	10	0.26
						7	T18	GATGCCAGAC	47	7	0.36
						8	OPC07	GTCCCGACGA	25	2	0.23
	Mean		50						42.25		

PB= Polymorphic Bonds; TNB= Total Number of Bonds; PIC= Polymorphic Information Content.

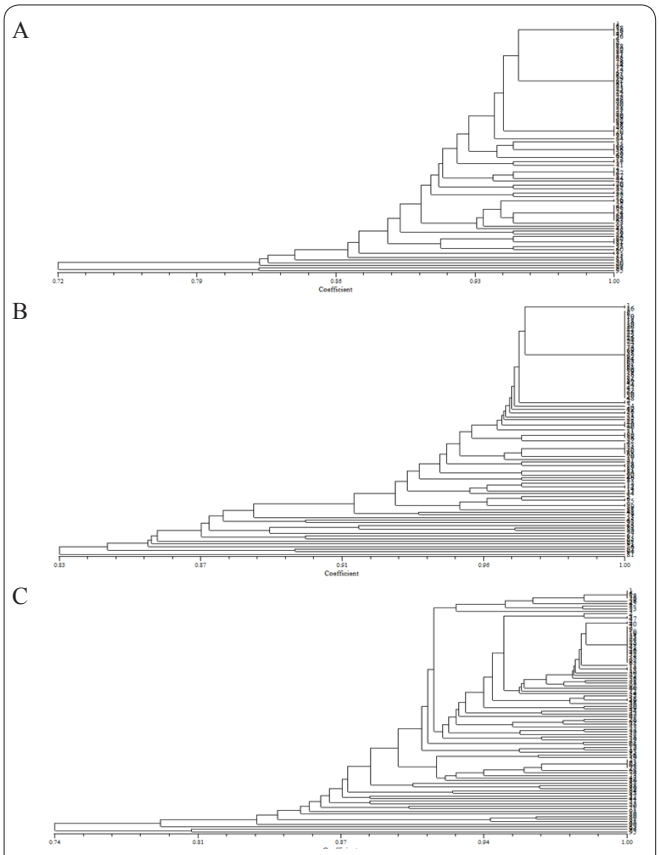

Figure 2. Dendrogram derived from a UPGMA cluster analysis of (A) ISSR, (B) RAPD and (C) RAPD+ ISSR markers using Jaccard' similarity coefficient of Chickpea mutants.

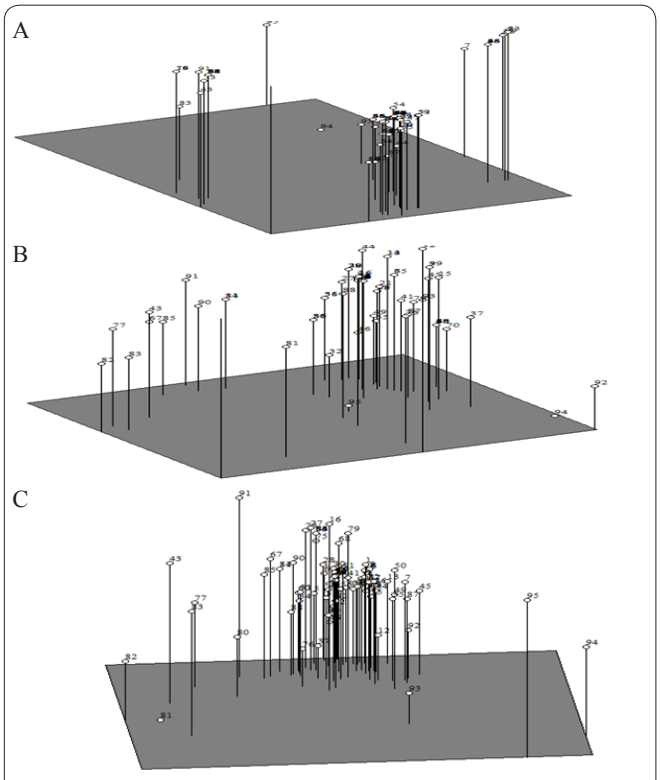

Figure 3. Plot of Chickpea mutants by principal coordinate analysis using the Jaccard's similarity coefficients (A) ISSR, (B) RAPD and (C) RAPD+ISSR markers.

In conclusion, mutagenesis via EMS induced genetic variations at suitable levels especially regards to agro morphological traits. This variation can be used as a genetic pool to selection for desire characteristics of chickpea as an important crop in Iran such as earliness, yield, blight resistance, wilt tolerance, herbicide tolerance and drought resistance.

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