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Assessment of Somaclonal Variations in Embryo-derived Axillary Shoots of Chickpea using Molecular Markers

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ABSTRACT

Background: Somaclonal variation is considered as a source of genetic variation for crop improvement. It has been investigated using cytological, biochemical and molecular techniques.

Methods: Genetic stability in the embryo-derived axillary shoots of 4 chickpea genotypes was assessed using eight inter-simple sequence repeat (ISSR) and 19 random amplified polymorphic DNA (RAPD) primers.

Result: RAPD primers produced 94 and ISSR primers produced 38 distinct and scorable alleles, with an average of 4.9 alleles for RAPD and 4.75 for ISSR primers. The polymorphic information content (PIC) ranged from 0.36 to 0.90 for RAPD and from 0.50 to 0.87 for ISSR. ISSR recognized a 90%, but RAPD recognized 82% similarity value. No absolute similarity value was between the mother plant and the regenerated shoots for the overall genotypes. At a 90% similarity value, 15 out of the 20 regenerated shoots from 'Giza 88' group with their mother plant using ISSR markers; however, 11 regenerated shoots grouped with their mother plant in one central cluster for 'Giza 4' using RAPD markers. The observed variations in the total number of polymorphic RAPD and ISSR bands and the number of bands specific to the mother and regenerated shoots, detected intra-clonal variation and genetic instability seem to be genotype-dependent.

Key words: Axillary shoots proliferation, Genetic fidelity, Intra-clonal variation, Molecular makers.

INTRODUCTION

Chickpea (*Cicer arietinum* L.; Fabaceae) contributes to human nutrition as it contains high protein content and β-carotene and minerals, such as phosphorus, calcium, magnesium, iron and zinc (Varshney *et al.* 2019). Advances in plant biotechnology, especially in cell and tissue culture techniques and molecular biology, provide valuable tools for managing and conservation of plant genetic resources. *In vitro* has been used to propagate, re-vegetation and genetic improvement of many leguminous species, including chickpea. It uses small amounts of original germplasm, production of pathogen-free propagules and shortening the time to generate new genotypes on a large scale (Aasim *et al.* 2011; Dewir *et al.* 2016; Kadiri *et al.* 2014; Ugandhar *et al.* 2012).

Regardless of the many benefits of direct regeneration through axillary shoots as a safe mode for producing true-to-type plants, the occurrence of genetic variability among the regenerated clones of one parental line is still risky (Larkin and Scowcroft, 1981). This variability could be because of chromosomal rearrangement, gene amplification, gene mutation and retrotransposon activation (Saker *et al.* 2000). Therefore, high genetic fidelity and true-to-type clones are critical for micro-propagation and to maintain the essential characteristics of the mother plant (Dewir *et al.* 2018). Somaclonal variations can be exploited to develop unfamiliar plant types. Several indicators, including phenotypic characteristics, chromosome number, isozyme profiles and PCR-based molecular markers, can assess genetic fidelity. Nowadays, molecular markers are the most popular tool for investigating the genetic fidelity of *in vitro*-regenerated plants. This study aimed to assess genetic fidelity in embryo-derived axillary shoots of four chickpea genotypes using random amplified polymorphic DNA (RAPD) and inter-simple sequence repeat (ISSR) markers.

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MATERIALS AND METHODS

Chickpea seeds of three Kabuli type genotypes ('Giza 4', 'Giza 195' and 'Giza 531') and one desi type genotype ('Giza 88') were washed with distilled water, surface-sterilized with 70% (v/v) ethanol for 10 s and then soaked in 20% (v/v) of a 5.2% sodium hypochlorite solution containing 2-3 drops of Tween 20 (polyoxyethylene-sorbitan monolaurate) for 15 min and then washing three times with sterile distilled water. After a 24 hr soak in sterile distilled water, twenty embryos of each cultivar were excised and cultured (Fig 1A) in five Magenta GA-7 culture vessels (77 × 77 × 97 mm; Magenta

LLC, Chicago, IL, USA) containing 60 mL basal (Murashige and Skoog) MS medium supplemented with 3% (w/v) sucrose, 0.8% (w/v) agar-agar and optimal concentrations of 6-benzyl amino purine (BAP) for each genotype to induce axillary shoot proliferation (*i.e.* 2 mg L^{-1} BAP for 'Giza 531' and 'Giza 88', 4 mg L^{-1} BAP for 'Giza 195' and 6 mg L^{-1} BAP for 'Giza 4'). The pH of the medium was adjusted to 5.8 and autoclaved at 121°C and 1.2 kg/cm psi for 15 min. All cultures were incubated for 15 days at 25°C under a 16 hours' photoperiod provided using cool-white fluorescent lights at 30 µmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD). In the second re-culture, twenty explants (clumps of shoots) from each genotype were randomly selected and used as plant material (Fig 1B) to study the occurrence of somaclonal variations.

Total genomic DNA was extracted from each young leaf tissue of the mother plant and embryo-derived axillary shoot clumps of each genotype tissue using SDS protocol. Quantification of the extracted DNA was determined by 1% agarose gel electrophoresis and DNA diluted with TE to a final concentration of 100 ng μL^{-1}. For each PCR, the 20 µL total reaction volume contained 1X Go-Taq green master mix (Promega; Madison, WI, USA). 0.1 µM each primer, 50 ng DNA template and nuclease-free water to complete the volume to 20 µL. The thermal cycler profile for PCR amplification was set as follows: denaturation at 94°C for 5 min; followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 33°C for 45 sec for RAPD primers and 55°C for ISSR primers and elongation at 72°C for 2 min; with a final elongation step at 72°C for 7 min and final storage at 4°C.

Amplified products of RAPD and ISSR were electrophoresed, stained with acridine dye and imagine using Gel Doc EZ™ 1708270 Bio-Rad (Fig 1C). Data were scored using a binary format (1 for presence and 0 for absence). The polymorphism information content (PIC) estimates according to Anderson *et al.* (1993). Jaccard's similarity coefficient (Jaccard 1908) was used to construct a dendrogram -the relationships among the genotypes and the genetic variability detected using the unweighted pair group method with an arithmetic average (UPGMA) employing the PAST software program V3.11 (Hammer *et al.* 2001).

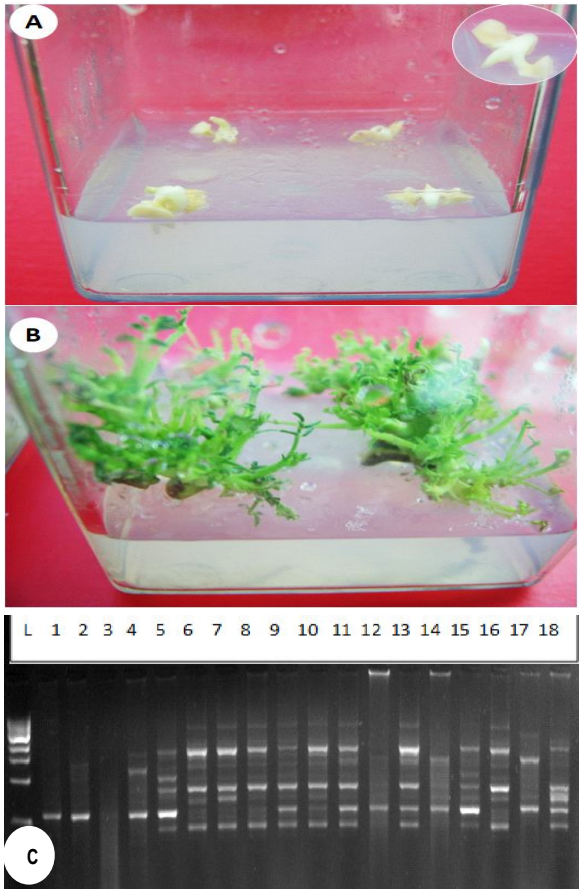


Fig 1: Establishment of chickpea *in vitro* culture (A) excised embryos explant used for culture initiation and (B) embryo-derived axillary shoot clumps used to study somaclonal variations. (C) An example of ISSR1 profile for Giza 88 and Giza 195 genotypes, L is DNA ladder.

RESULTS AND DISCUSSION

Out of the 80 RAPD and 16 ISSR primers screened, only 19 RAPD and 8 ISSR primers produced precise, reproducible and scorable alleles. The number of amplified bands for each primer is shown in Tables 1 and 2. The 19 RAPD primers produced 94 and ISSR primers produced 38 distinct and scorable alleles, with an average of 4.9 alleles produced per RAPD primer and 4.75 alleles per ISSR primer across genotypes. The number of scorable alleles varied from 2 (RAPD primers, OP-A2 and OP-F13 and ISSR8) to 10 (RAPD; OP-F10 and OP-F20) and 8 for primer ISSR5. Moreover, the number of polymorphic alleles varied across genotypes, with an average of 1.0 to 1.9 polymorphic alleles for 'Giza 4' and 'Giza 531' using RAPD; however, except primers ISSR1, ISSR5 and ISSR6, all banding profiles from micro-propagated plants were monomorphic and consistent with those of the mother plant using ISSR markers. The polymorphism percentage ranged from 0.0 to 100% for both 'Giza 4' and 'Giza 531' using RAPD markers; however, it ranged from 0.0 to 100% in 'Giza 531' using ISSR markers. In the RAPD profile, the PIC values ranged from 0.36 for primer OP-K16 to 0.90 for OP-F10 and OP-F20. Six RAPD primers showed a monomorphic pattern across all genotypes. However, seven primers showed a monomorphic pattern for at least one genotype and six primers generated a polymorphic pattern across all genotypes. In ISSR markers, PIC was consistent across primers and genotypes; it ranged from 0.50 across genotypes to 0.87 in 'Giza 4' and 'Giza 531' genotypes.

RAPD and ISSR markers were selected because of their simplicity and cost-effectiveness. The use of two types of markers, which amplify different regions of the genome, allows for a better analysis of genetic stability/variation of the plantlets (Yuan *et al.* 2009). The clonal fidelity assessment

Table 1: List of primers, sequences, number of the amplified fragments, polymorphism % and the PIC value got using RAPD primers in embryo-derived axillary shoots of chickpea genotypes.

Primer 5′-3′		Chickpea genotype											
		'Giza 195'			'Giza 4'			'Giza 531'			'Giza 88'		
		Alleles	Poly	PIC	Alleles	Poly	PIC	Alleles	Poly	PIC	Alleles	Poly	PIC
OP-A2	TGCCGAGCTG	2	0.0	0.50	2	0.0	0.50	2	0.0	0.50	2	0.0	0.50
OP-A9	GGGTAACGCC	4	50.0	0.68	4	50.0	0.68	4	50.0	0.74	4	75.0	0.70
OP-F9	CCAAGCTTCC	7	0.0	0.86	7	0.0	0.86	7	85.7	0.86	7	71.4	0.86
OP-F10	GGAAGCTTGG	10	70.0	0.90	10	80.0	0.90	10	90.0	0.90	10	60.0	0.90
OP-F13	GGCTGCAGAA	2	0.0	0.50	2	0.0	0.50	2	0.0	0.50	2	0.0	0.50
OP-F14	TGCTGCAGGT	3	33.3	0.67	3	0.0	0.67	3	0.0	0.67	3	0.0	0.67
OP-F3	CCTGATCACC	3	66.7	0.66	3	100.0	0.66	3	100.0	0.67	3	33.3	0.67
OP-F4	GGTGATCAGG	6	0.0	0.83	6	0.0	0.83	6	0.0	0.83	6	0.0	0.83
OP-F12	ACGGTACCAG	4	25.0	0.67	4	25.0	0.69	4	50.0	0.75	4	75.0	0.70
OP-F15	CCAGTACTCC	7	0.0	0.86	7	0.0	0.86	7	0.0	0.86	7	0.0	0.86
OP-F20	GGTCTAGAGG	10	0.0	0.90	10	0.0	0.90	10	50.0	0.90	10	0.0	0.90
OP-K7	AGCGAGCAAG	3	0.0	0.67	3	0.0	0.67	3	0.0	0.67	3	0.0	0.67
OP-K12	TGGCCCTCAC	3	33.3	0.45	3	0.0	0.50	3	66.7	0.53	3	33.3	0.66
OP-K16	GAGCGTCGAA	4	75.0	0.67	4	25.0	0.59	4	25.0	0.36	4	25.0	0.66
OP-K17	CCCAGCTGTG	4	50.0	0.75	4	75.0	0.73	4	75.0	0.75	4	50.0	0.75
OP-E18	GGACTGCAGA	6	0.0	0.80	6	0.0	0.75	6	33.3	0.82	6	16.7	0.83
OP-G20	TCTCCCTCAG	5	80.0	0.77	5	20.0	0.76	5	0.0	0.75	5	80.0	0.79
OP-C11	AAAGCTGCGG	5	80.0	0.80	5	0.0	0.80	5	40.0	0.79	5	60.0	0.79
OP-G19	GTCAGGGCAA	6	0.0	0.83	6	0.0	0.83	6	0.0	0.83	6	0.0	0.83
Total		94	-	-	94	-	-	94	-	-	94	-	-
Average		4.9	29.64	0.72	4.9	19.74	0.72	4.9	35.03	0.72	4.9	30.51	0.74
Min		2.0	0.0	0.45	2.0	0.0	0.50	2.0	0.0	0.36	2.0	0.0	0.50
Max		10.0	80.0	0.90	10.0	100.0	0.90	10.0	100.0	0.90	10.0	80.0	0.90

Table 2: List of primers, sequences, number of the amplified fragments, polymorphism % and the PIC value got using ISSR primers in embryo-derived axillary shoots of chickpea genotypes.

Primer 5'-3'		Chickpea genotype											
		'Giza 195'			'Giza 4'			'Giza 531'			'Giza 88'		
		Alleles	Poly	PIC	Alleles	Poly	PIC	Alleles	Poly	PIC	Alleles	Poly	PIC
ISSR1	(AAC) 7C	7	71.43	0.86	7	42.85	0.82	7	42.85	0.82	7	57.14	0.81
ISSR2	(AAC) 7A	5	0	0.80	5	0	0.80	5	0	0.80	5	0	0.80
ISSR3	(AAC) 7T	3	0	0.67	3	0	0.67	3	0	0.67	3	0	0.67
ISSR4	(GTT) 7A	5	0	0.80	5	0	0.80	5	0	0.80	5	0	0.80
ISSR5	(GTT) 7T	8	62.5	0.78	8	75	0.87	8	37.5	0.87	8	37.5	0.84
ISSR6	(CAC) 7G	4	25	0.67	4	25	0.68	4	100	0.68	4	0	0.75
ISSR7	(TGT) 7G	4	0	0.75	4	75	0.75	4	0	0.75	4	0	0.75
ISSR8	(TGT) 7C	2	0	0.50	2	0	0.50	2	0	0.50	2	0	0.50
Total		38	-	-	38	-	-	38	-	-	38	-	-
Average		4.75	29.05	0.74	4.75	27.33	0.74	4.75	22.54	0.74	4.75	11.83	0.74
Min		2	0	0.5	2	0	0.5	2	0	0.5	2	0	0.50
Max		8	71.43	0.86	8	75	0.87	8	100	0.87	8	57.14	0.84

using two types of DNA markers in earlier works showed similar results (Mukhopadhyay *et al.* 2016). ISSRs are widely distributed throughout the genomic DNA and make the amplification of genomic DNA in a particular region. Moreover, the higher amplification efficiency of ISSRs makes genetic inferences much clearer and provides clues for genetic variation at inter- and even intra-specific levels (Faisal *et al.* 2018). In this study, RAPD markers generated a considerably high polymorphism percentage that varied among genotypes and ranged from 19.74% for 'Giza 4' to 35.03% for 'Giza 531'. However, ISSR generated a polymorphism percentage that ranged from 11.83% in 'Giza 88' to 29.0% in 'Giza 195'. This percentage indicated high variability among the regenerated shoots as compared with mother plants. The differences between these markers could be due to that the two techniques targeted different portions of the genome, highlighting the importance of the number of loci and their coverage of the overall genome for obtaining reliable estimates of genetic relationships.

Somaclonal and developmental variations are considered a useful source of variation to the plant breeders and a possible means for inducing genetic variability in crop plants. They occur due to pre-existing variations in the somatic cells of the explant (genetic influence) or could be generated during tissue culture (epigenetic influence); these variations are expected to generate stable plants carrying attractive heritable traits (Larkin and Scowcroft, 1981). The source and age of explants, culture duration, number of sub-cultures, culture environment (temperature and pH). Chemical additives, growth stimulants or regulators, medium composition, level of ploidy and genetic mosaicism are factors responsible for variability *in vitro* (Goto *et al.* 1998; Devi *et al.* 2013).

The mechanism of somaclonal variation has been studied. These variations may display as cytological abnormalities, frequent qualitative and quantitative phenotypic mutations, sequence changes, gene activation and silencing (Sheidai *et al.* 2008). Hence, the assessment of genetic fidelity of the regenerates is a critical parameter for the establishment of an efficient regeneration protocol. Molecular markers are considered a powerful tool for monitoring the occurrence of somaclonal variations and ensuring the genetic fidelity of micropropagated plants; thus, identifying genotypes with optimal response to *in vitro* culture conditions the growth stage or the environmental conditions (Chhajer and Kalia, 2016).

The occurrence of somaclonal variations among the micropropagated plantlets of *Malus pumila* (Modgil *et al.* 2005) and *Platanus acerifolia*, using RAPD or ISSR markers, were reported (Huang *et al.* 2009). In contrast, Kumar *et al.* (2010) employed 30 RAPD and 12 ISSR primers exhibited similar banding patterns across all the micropropagated plants using both RAPD and ISSR markers and concluded that the developed micropropagation protocol was appropriate for the true-to-type clonal propagation of the date palm. El-Awady *et al.* (2015) reported that 12 RAPD

and 9 ISSR primers employed to Rhazya stricta Decne plantlets generated 5.2% and 5.8% polymorphism percentages. In almonds, 64 RAPD and 10 ISSR primers generated no polymorphic bands (Martins *et al.* 2005). They conclude that the studied regenerates possess high genetic stability and that the protocols used seem to be suitable for maintaining the integrity of the genome. Moreover, ISSR markers were used to confirm the genetic homogeneity in mulberry plants and its plantlets showed clonally uniform and genetically stable (Rohela *et al.* 2018).

Based on the similarity coefficients generated from the ISSR and RAPD data of the scored bands. For 'Giza 88' and its derivatives, 15 out of the 20 axillary shoots could be grouped with its mother into a single cluster with 90% similarity using ISSR markers (Fig 2A). Using RAPD markers, most of the *in vitro* generated plants formed one central cluster composed of 14 plantlets at an 83% similarity level (Fig 2B). Furthermore, Fig 3A shows that 'Giza 195' and it's *in vitro* derivatives generated more polymorphisms; 10 out of the 20 axillary shoots could be grouped with their mother into a single cluster, with a 90% level of similarity using ISSR markers. RAPD profile at 86% similarity showed 'Giza 195' grouped with ten regenerated shoots, whereas the remaining genotypes were distributed into three clusters. (Fig 3B). *In vitro* derivatives of 'Giza 4' and its mother showed a range of similarity between 60-100% based on ISSR profile (Fig 4A). Specifically, 11 out of the 20 axillary shoots grouped with their mother into a single cluster with a 90% level of similarity; however, at 85% of similarity, most of the regenerated shoots could be grouped with their mother. RAPD profile for 'Giza 4' showed at 90% similarity, 11 regenerated shoots could be grouped with their mother into one central cluster. (Fig 4B). Twelve out of the 20 shoots of 'Giza 531' and its derivatives could be grouped with their mother into a single cluster with a 90% level of similarity based on ISSR data (Fig 5A). RAPD profile at an 82% similarity level, 16 of the platelets derived from 'Giza 531' and their mother formed a central cluster (Fig 5B).

The observed variations in the total number of polymorphic RAPD and ISSR bands and the number of specific bands among the mother and regenerated plants indicate genetic differences of the genotypes due to induced somaclonal variations. Sheidai *et al.* (2008) reported that the presence of bands in mother plants and the absence of the same bands in the regenerated banana plants (*Musa acuminate*) indicate the loss of particular loci during tissue culture due to somaclonal variation. In contrast, the occurrence of bands in the regenerated plants and absence

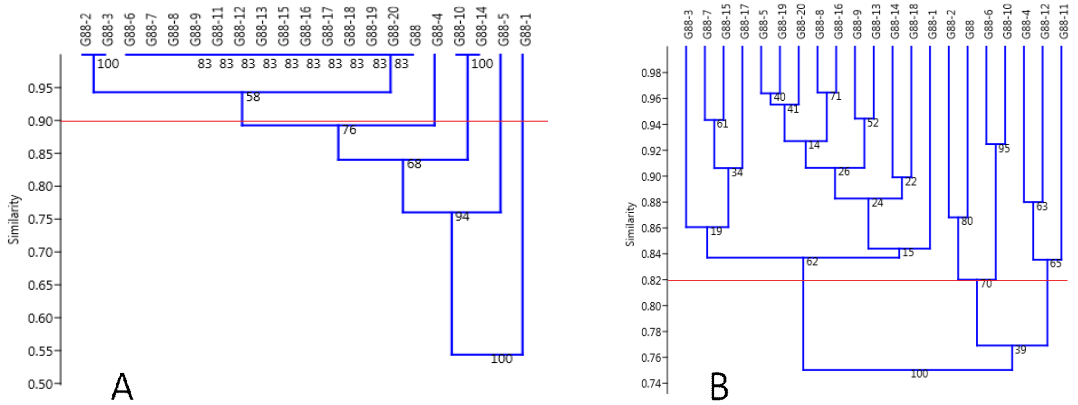


Fig 2: UPGMA cluster analysis for the 'Giza 88' mother and its 20 regenerated axillary shoots analyzed by ISSR (A) and RAPD (B) markers.

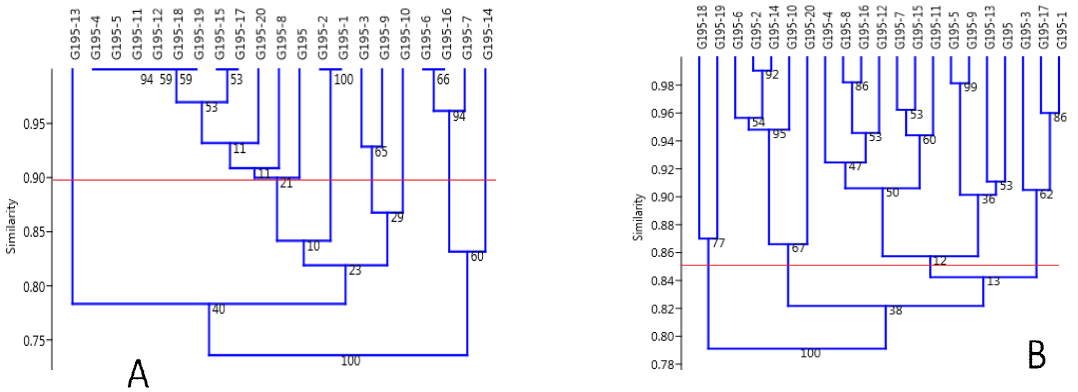


Fig 3: UPGMA cluster analysis for the 'Giza 195' mother and its 20 regenerated axillary shoots analyzed by ISSR (A) and RAPD (B) markers.

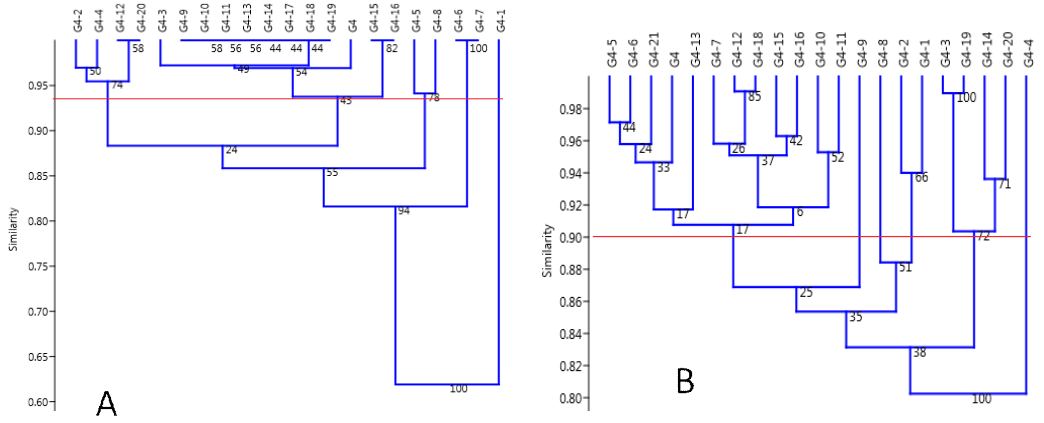


Fig 4: Dendrogram obtained using UPGMA cluster analysis for the 'Giza 4' mother and its 20 regenerated axillary shoots analyzed by ISSR (A) and RAPD (B) markers.

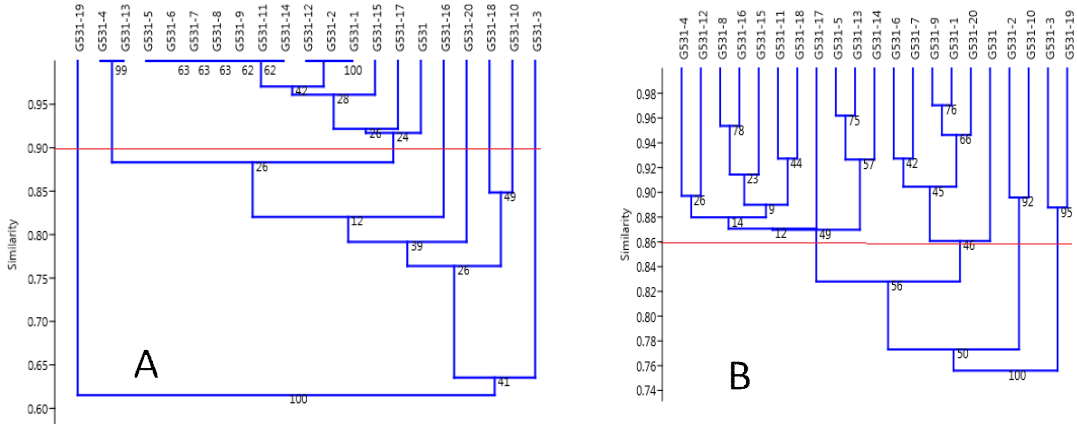


Fig 5: Dendrogram obtained using UPGMA cluster analysis for the 'Giza 531' mother and its 20 regenerated axillary shoots analyzed by ISSR (A) and RAPD (B) markers.

in mother plants may indicate the occurrence of genetic changes leading to the formation of new binding sites in these plants. However, the importance of these bands in the genetic identification of the genotypes or somaclones is well-documented (Venkatachalam *et al.* 2007). The observed variations in the total number of polymorphic RAPD and ISSR bands and the number of specific bands among the mother and regenerated plants indicate genetic differences of the genotypes due to induced somaclonal variations. The number of specific bands among the mother and regenerated shoots showed intra-clonal variation and instability seems to be genotype-dependent. Roy *et al.* (2001) first reported the generation of variability in chickpea through tissue culture, showed that phenotypic variability among the micropropagated plants concerning seed size and seed coat color had been observed.

CONCLUSION

The results obtained in the present study open the possibility of developing new plant types through the exploitation of somaclonal variations and both ISSR and RAPD showed the power to detect the variation generated from *in vitro* culture.

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Conflicts of interest

The authors declare no conflict of interest.

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