

Original Research Article

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Assessment of Genetic Fidelity of Micropropagated Gerbera (*Gerbera jamesonii* Bolus) Plants by ISSR Markers

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ABSTRACT

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The genetic fidelity of *in vitro* raised gerbera plants multiplied through micropropagation upto fifteen *in vitro* subcultures was assessed by using inter-simple sequence repeat (ISSR) markers. 40 ISSR primers screened, 20 ISSR primers produced a total of 54 ISSRs clear, distinct and reproducible amplicons, which were monomorphic across all micropropagated plants (15) studied. Thus, a total 1094 bands were generated which exhibited homogeneous banding patterns with ISSR markers. All banding profiles from micropropagated plants were monomorphic and similar to those of the mother plant. These results indicate that the micropropagation protocol developed by us for rapid *in vitro* multiplication is appropriate and suitable for clonal propagation gerbera.

Introduction

Gerbera (*Gerbera jamesonii* Bolus) is considered as one of the nature's beautiful creations because of having excellent flowers of exquisite shape, size and bewitching colour. It is native to South Africa, popularly known as 'Transval daisy' or 'Barbeton daisy'. Gerbera ranks fifth in the global cut flower trade. It is a popular cut flower in Holland, Germany and USA. Gerbera is ideal for beds, borders, pots and rock gardens. The flowers are of various colours, suit very well in different floral arrangements and decorations.

The cut blooms also have a long vase life of about 7 to 8 days. Micropropagation from different explants such as shoot tips, capitulum and leaf segments is a viable approach for large-scale multiplication of gerbera (Aswath and Choudhary, 2002). Gerbera produces stem less herbs with radical, etiolated leaves, which are entire or sometimes lobed. The foliage in some species is entire and have lighter under surface. Flower heads are solitary, multi-flowered with conspicuous ray florets in 1 or 2 rows. The disc flowers may be numerous to almost absent. Disc florets can be yellow, the same colour as the

rays, or vividly contrasting black. Bicolours also exist (Tourje *et al.*, 1994). Based on the flower heads, they may be grouped into single, semi-double and double cultivars (Loeson, 1986). The showy flower heads, in almost every color except blue and purple are carried on bare stems 18 inch 45 centimeter (cm) long.

Gerbera is commercially multiplied through tissue culture using shoot tip explant and subculturing frequently. This method allows for obtaining large amounts of healthy homogenous plants. However, frequent subculture in cytokinin and auxin media leads to vitrification, loss of vigor and production of somoclonal aberration. Moreover, frequent sub-cultures in gerbera may lead to high cost in operations. It is therefore, imperative to establish genetic uniformity of micropropagated plants to conform the quality of the plantlets for its commercial utility.

In any micropropagation protocol, assessment of genetic stability of the plantlets helps to know the true-to-type nature of the plantlets or variability induced in tissue culture cycle. The occurrence of cryptic genetic changes arising via somaclonal variation in the regenerants can seriously limit the broader utility of the micropropagation system (Salvi *et al.*, 2001). A major problem associated with micropropagation is occurrence of somaclonal variation among the sub-clones parental lines, arising as a direct consequence of *in vitro* culture of plant cells, tissues and organs. The frequency of these variations varies with the source of explant and their pattern of regeneration (somatic embryogenesis/organogenesis/ axillary bud multiplication), media composition and culture conditions (Damasco *et al.*, 1996; Salvi *et al.*, 2001). It is therefore, imperative to establish genetic uniformity of micropropagated plants to conform the quality of the plantlets for its commercial utility.

Clonal fidelity is of major importance in micropropagation of any genotype or advanced line which is commercially important (Gupta and Varshney, 2009). Since the possibility of genetic aberration is a common occurrence in any tissue culture venture, monitoring the genetic fidelity among the regenerates is an important prerequisite prior to extensive cultivation. Normally during regeneration by using shoot tip explant and subculturing frequently is likely chance to get somoclonal variation. So it is advised to subject these plants for genetic fidelity test by using ISSR markers.

Polymerase chain reaction (PCR) based technique called inter simple sequence repeats (ISSR) are immensely useful in establishing the genetic stability of *in vitro* regenerated plantlets in many crop species (Lakshmanan *et al.*, 2007; Joshi and Dhawan, 2007). Evaluation of genetic fidelity of *in vitro* propagated gerbera using RAPD and ISSR markers was attempted by Bhatia *et al.*, (2010). Capitulum regenerated plants were subjected to molecular characterization and they observed monomorphic banding pattern in micropropagated plants which were similar to those of mother plant. The main advantage of ISSR (Inter Simple Sequence Repeat) markers is very simple, fast, cost-effective, highly discriminative and reliable (Leroy *et al.*, 2001). They require only a small quantity of DNA sample (5-50ng per reaction) and they do not need any prior sequence information to design the primer. They do not use radioactive probes as in restriction fragment length polymorphism (RFLP). ISSRs are largely distributed throughout the genome. Thus, they are suitable for the assessment of the genetic fidelity of *in vitro* regenerated plants.

In this study, A set of 15 *in vitro* raised clones derived from three different *in vitro* subcultures from 13-15th subculture plants along with mother plant were selected for the

assessment of genetic fidelity by using ISSR markers. This is very useful for establishing a particular micropropagation system for the production of genetically identical and stable plants before it is released for commercial purposes.

Materials and Methods

Plant material

Gerbera variety Arka Ashwa used in the present study was grown under polyhouse conditions at Division of Floriculture and Medicinal crops, ICAR- IHR, Bengaluru. The rooted plantlets were hardened on coco peat and soil rite mixture. A set of 15 *in vitro* raised clones derived from three different *in vitro* subcultures from 13-15th subculture plants along with mother plant were randomly selected for assessment of clonal fidelity by ISSR markers.

DNA extraction and PCR amplification condition

Genetic fidelity of *in vitro* raised clones were tested using ISSR markers. For this purpose 15 *in vitro* raised clones derived from three different *in vitro* subcultures were chosen randomly and compared with mother plant from which explants were taken. Isolation of good quality genomic DNA is one of the most important pre requisites for doing ISSR analysis. The CTAB procedure reported by Doyle and Doyle (1987) was used for the extraction of good quality genomic DNA from leaf tissue. CTAB was used to liberate the nucleic acid from cell, which was then further purified phenol chloroform to remove proteins and other contaminating plant debris. Good quality genomic DNA (30 to 50ng/μl) isolated from gerbera leaf samples of plantlets regenerated from buds and leaf explants (direct and indirect organogenesis) were subjected to ISSR assay. ISSR primers

supplied by 'Sigma Aldrich Chemical Pvt. Ltd.' with good resolving power were used for amplification of DNA. Forty ISSR primers were used for initial screening. PCR amplification was carried out in a total volume of 20 μl containing 2 μl (30 ng) of genomic DNA. The reaction buffer consisted of 2 μl of 10X *Taq* assay PCR buffer, 2 μl MgCl₂ (2 mM), 1.5 μl dNTPs (10 mM each), 1.5 μl primer, 0.4 μl DNA *Taq* polymerase and 10.6 μl water. The primers showing polymorphic bands were then used for analysing the clonal fidelity of micropropagated plants. PCR amplification was performed in a DNA thermal cycler which was programmed for initial DNA denaturation at 94°C for 4 min, followed by 35 cycles of 1 min denaturation at 94°C, 1 min annealing (temperature specific to the primer) and 2 min extension at 72°C, with a final extension at 72°C for 8 min. Amplified products were resolved by electrophoresis on 1.5% agarose gel using 1X TAE buffer stained with ethidium bromide along with marker (λDNA/*EcoRI*+*HindIII* double digest). The profile was visualized under UV transilluminator and documented using gel documentation. The documented ISSR profiles were carefully examined for monomorphism.

Data scoring and analysis

ISSR assay was performed with DNA samples isolated from 16 different sources and micropropagated plants derived from capitulum *in vitro* sub cultured using forty primers. Consistent, well-resolved fragments in the size range of 100 bp to 1 kb were manually scored. Each band was treated as a marker. The scoring of bands was done on the basis of their presence ('1') or absence ('0') in the gel and missing data was denoted by '9'. Clustering was done based on Jaccards similarity matrices data by applying Unweighted Pair Group Method with Arithmetic averages (UPGMA) and cluster

analysis was carried out by Darwin 6.0 pc software.

Results and Discussion

A total of 40 ISSRs used in the initial screening, only twenty ISSRs produced clear and reproducible PCR products were selected for amplification of samples. The base sequence of these ISSRs primers is given in the Table 1. The optimum annealing temperature for ISSR markers varied from 41.9°C to 59.5°C. The twenty ISSRs (Table 2) produced 54 distinct and scorable bands in the size range of 100 bp (ISSR-809, ISSR-844, ISSR-857, ISSR-861, ISSR-862 and ISSR-891) to 1650 bp (ISSR-889). The number of scorable bands for each ISSR varied from 1 (ISSR-808, ISSR-816, ISSR-847, ISSR-855 and ISSR-888) to 7 (ISSR-857), with an

average of 5.14 bands per primer. The PCR amplified profiles from fifteen micropropagated plants were monomorphic and similar to those of the mother plant. This study report on the use of ISSR-PCR, to assess the genetic fidelity of *in vitro* raised plants of gerbera derived from three different sub cultures, viz., subculture-13, subculture-14 and sub-culture 15 (five regenerants in each subculture) by using 40 ISSR markers, for. Out of 40 ISSRs, 20 ISSRs generated a total of 54 bands, all were monomorphic (Plate 1 and 2) across the clones of gerbera. The banding profiles among the *in vitro* propagated plants were monomorphic and similar to those of the mother plant. The clone derived from mother plant and *in-vitro* raised explants of different subcultures show no genetic variation. This confirmed the true-to-type nature of the *in- vitro* raised clones.

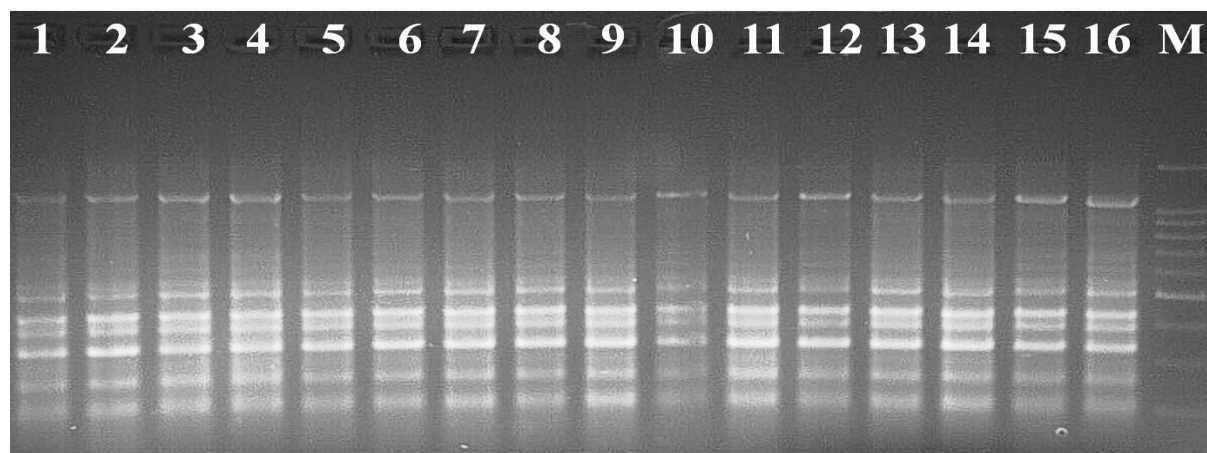
Table.1 List of ISSR primers, their sequence motifs and annealing temperatures

Sl. No.	Primers	Nucleotide Sequence (5'-3')	Annealing temperature(°C)
1	ISSR-808	5'-AGAGAGAGAGAGAGAGC-3'	50.8
2	ISSR-809	5'-AGAGAGAGAGAGAGAGG-3'	50.8
3	ISSR-816	5'-CACACACACACACACAT-3'	42.9
4	ISSR-828	5'-TGTGTGTGTGTGTGTGA-3'	41.9
5	ISSR-844	5'-CTCTCTCTCTCTCTCTRC-3'	44.9
6	ISSR-845	5'-CTCTCTCTCTCTCTCTRG-3'	43.8
7	ISSR-846	5'-CACACACACACACACART-3'	50.5
8	ISSR-847	5'-CACACACACACACACARC-3'	44.9
9	ISSR-848	5'-CACACACACACACACARG-3'	44.9
10	ISSR-855	5'-ACACACACACACACACYT-3'	59.5
11	ISSR-857	5'-ACACACACACACACACYG-3'	45.1
12	ISSR-858	5'-TGTGTGTGTGTGTGTGRT-3'	46
13	ISSR-859	5'-TGTGTGTGTGTGTGTGRC-3'	48.2
14	ISSR-861	5'-ACCACCACCACCACCACC-3'	59.5
15	ISSR-862	5'-AGCAGCAGCAGCAGCAGC-3'	59.5
16	ISSR-866	5'-CTCCTCCTCCTCCTCCTC-3'	53
17	ISSR-888	5'-BDBCACACACACACACA-3'	42.9
18	ISSR-889	5'-DBDACACACACACACAC-3'	42.9
19	ISSR-890	5'-VHVGTGTGTGTGTGTGT-3'	42.9
20	ISSR-891	5'-HVHTGTGTGTGTGTGTG-3'	42.9

Table.2 Number and size of the amplified fragments generated by ISSR primers in gerbera

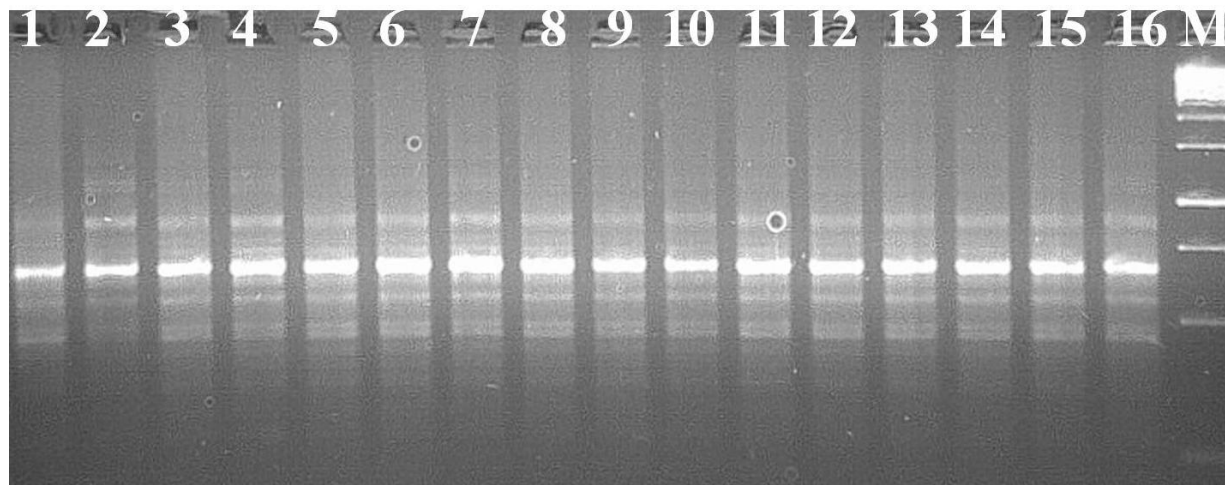
Sl. No.	Primers	Number of scorable PCR products per ISSR	Total number of PCR amplified products	Size range (bp)
1	ISSR-808	1	38	140-1030
2	ISSR-809	5	102	100-1110
3	ISSR-816	1	33	240-510
4	ISSR-828	2	54	400-900
5	ISSR-844	4	50	100-560
6	ISSR-845	2	61	170-1110
7	ISSR-846	5	43	600-1270
8	ISSR-847	1	55	250-1170
9	ISSR-848	4	53	170-370
10	ISSR-855	1	42	150-570
11	ISSR-857	7	119	100-1010
12	ISSR-858	2	58	180-370
13	ISSR-859	2	45	110-470
14	ISSR-861	2	34	100-380
15	ISSR-862	5	49	100-930
16	ISSR-866	3	49	150-690
17	ISSR-888	1	44	120-1110
18	ISSR-889	2	76	460-1650
19	ISSR-890	2	40	300-1240
20	ISSR-891	2	49	100-570
Total		54	1094	
Mean		5.14	104.19	

Plate.1 Amplification pattern of primer ISSR-857



Mother plant: 1, Regenerants 2-6: 13th subcultured plants, 7-11: 14th subcultured plants, 12-16: 15th subcultured plants, M: Marker Lambda DNA (*EcoRI/Hind* III digest 1000bp)

Plate.2 Amplification pattern of primer ISSR-848\



Mother plant: 1, Regenerants 2-6: 13th subcultured plants, 7-11: 14th subcultured plants, 12-16: 15th subcultured plants, M: Marker Lambda DNA (*EcoRI/Hind* III digest 1000bp)

The ISSR technique is a PCR-based method, which involves amplification of DNA segment present at amplifiable distance in between two identical microsatellite repeat regions oriented in opposite direction (Reddy *et al.*, 2002). The technique uses microsatellite, usually 16–25 bp long as primer in single PCR reaction targeting multiple genomic loci to amplify mainly the inter-SSR sequences of different sizes.

The genetic fidelity reveals that there is no genomic variation observed at DNA/molecular level from the plantlets obtained from different *in vitro* sub cultured plants. The results were similar to the findings of Bhatia *et al.*, (2010), where in genetic fidelity of *in vitro* raised plants of gerbera derived from three different explants *viz.*, capitulum, leaf and shoot tips using ISSR assay. The clones derived from capitulum and shoot tip explants did not show any genetic variation, whereas one of the leaf-derived clones exhibited some degree of variation. Bhatia *et al.*, (2011) assessed genetic fidelity of *in vitro*-raised gerbera derived from capitulum explants by using RAPD and ISSR markers and reported that all banding profiles from micropropagated plants were

monomorphic and similar to those of the mother plant.

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