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Analysis of Somaclonal Variation using Molecular Markers in *in vitro* Regenerated *Chrysanthemum* cv. Pusa Centenary

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A B S T R A C T

Detection of somaclonal variation in tissue cultured plants is essential for conservation of elite varieties and also to avoid undesired characters. The genetic fidelity of *in vitro* regenerated *Chrysanthemum* cv. Pusa centenary, a commercially important cultivar was assessed using RAPD and ISSR markers. Regeneration was obtained via organogenesis and somatic embryogenesis from leaf and petal explants respectively. Murashige and Skoog (MS) media with different plant growth regulators (PGR) were used for *in vitro* experiments with leaf explants. Among the different PGR combinations used in MS media, 2,4-Dichlorophenoxyacetic acid (2,4-D) (1 mg L^{-1}) and ά-naphthalene acetic acid (NAA) (1 mg L^{-1}) along with 0.5 mg $L^{-1}$of 6-benzylaminopurine (BAP) or Kinetin (Kin) was optimal for producing 83-93% callus induction from leaf explants. On transfer to light on MS media with BAP (0.5 mg L^{-1}) and reduced auxins, the calli started turning green and proliferating into plantlets. Petal explants on the same media, produced direct somatic embryos within 10-14 days. On subculture, profuse production of somatic embryos with root and shoot poles was obtained. Nineteen primers (9 RAPD and 10 ISSR primers) produced 825 clear reproducible and scorable bands. While RAPD primers produced completely monomorphic bands with both leaf and petal regenerated plants in comparison to their mother plant, it was found that ISSR primers were able to detect variation in leaf callus derived plantlets. The plants regenerated via organogenesis from leaf explants were showing a variation of 0.66% with ISSR primers and plants regenerated from distinct somatic embryos of petal explants showed completely monomorphic bands with both RAPD and ISSR proving it's true to type nature. These results indicate that *in vitro* culture process induces rearrangements at the DNA level and demonstrates discrepancies between the pathways involved in regeneration.

Introduction

Chrysanthemum is globally the second economically important floriculture crop followed by rose and one of the most important ornamental species (Teixeira da Silva, 2004). In *Chrysanthemum* it is reported that *in vitro* techniques are useful to obtain plants that are free of Tomato aspermy virus, Cucumber mosaic virus and *Chrysanthemum* B virus (Ram *et al.*, 2009). Also *in vitro* propagation is a useful strategy to get large number of plants from limited tissues which can meet the demand in the expected time.

Chrysanthemum Pusa centenary is a newly released variety which is a gamma ray induced mutant of Thai Chen Queen. This cultivar is in demand due to its vigorous growing nature and it produces yellow flowers which remain fresh for 20 - 22 days in the field as well as in vase. To meet the demand we attempted to develop *in vitro* protocols for large scale multiplication of this cultivar from leaf and petal explants. Though protocols for *in vitro* regeneration are reported in *Chrysanthemum* (Himstedt *et al.,* 2001; Petty *et al.,* 2003; Waseem *et al.,* 2009) a varied response of genotypes to auxins and cytokinins are seen from these reports and the need to develop cultivar specific protocols have been emphasized (Song *et al.,* 2011). They also accentuate that establishment of propagation protocols must be optimized for each commercially important cultivar.

Retaining the genetic integrity of micropropagated plants is a major concern in tissue culture. Generally the problem associated with *in vitro* culture is the occurrence of genetic variation resulting from micropropagation i.e. somaclonal variation amongst sub-clones of one parental line arising as a direct consequence of *in vitro* culture of cells, tissues and organs (Larkin and Scowcroft, 1981; Gould, 1986). Hence a quality checkup for true to type plants at an early stage is very essential (Zilberman and Henikoff, 2007). Molecular markers like Random Amplified Polymorphic DNA (RAPD) and Inter-Simple Sequence Repeats (ISSR) have been suggested to be useful for confirmation of genetic fidelity in micropropagated plants (Ray *et al.,* 2006). These two techniques have the advantages of giving reproducible results, low cost and primers can be designed without any prior knowledge of sequences (Pradeep *et al.,* 2002). RAPD is a simple, dominant, quick and easy assay marker which requires low quantities (5-50ng) of template DNA. ISSR technique is also fast, simple and cost effective and a highly reliable technique which uses a single sequence repeat motif as a primer for amplification of regions between microsatellites. This paper reports differential response of leaf and petal explants under *in vitro* conditions for plant regeneration and the use of RAPD and ISSR techniques for assessing the genetic homogeneity of the regenerated plants from both the explants.

Materials and Methods

Experiment with leaf explants

Plant material

Leaves were collected from a single mature plant of Pusa centenary which was maintained in the glass house. They were washed thoroughly in running tap water for two hours followed by washing with tween 20 (2drops per 100ml of water). Surface sterilization was done by immersion in 0.1% mercuric chloride for 5 minutes followed by three washes with double distilled sterile water. They were cut into fine pieces of 3-4mm long sections and placed on the culture media with the adaxial surface touching the media.

Culture media and growth conditions for callus induction

The basal media used was MS (Murashige and Skoog, 1962) basal salts supplemented with sucrose (3%), and agar (0.8%), (Himedia, Mumbai, India). For callus induction MS basal media was supplemented with 2,4-Dichlorophenoxyacetic acid (2,4-D) (2 mg L^{-1}) and ȁ-naphthalene acetic acid (NAA) (2 mg L^{-1}) singly and in combination as 2,4-D, (1 mg L^{-1}) and NAA (1mg L^{-1}) along with cytokinins like 6-benzylaminopurine(BAP) or Kinetin at 0.5 mg L^{-1}. Six different combinations of plant growth regulators in MS basal were prepared and coded as M1 to M6

(Table 1). These media was solidified with agar @ 8 g L^{-1} (Himedia). For all media prepared the pH was adjusted to 5.8 with either 0.1N NaOH or 0.1 N HCl and they were dispensed in test tubes (Borosil make 25X150mm) and each test tube contained 20ml media. They were sterilized at 121^0C at 1.06 kg/cm^2 for 15 minutes. Explants were cultured in test tubes at the rate of one explant per tube. A total of 27 explants were inoculated in each treatment and maintained as three replications of nine explants each. Explants were cultured in dark at 25 ± 2°C for callus induction for 1 month. The number of cultures producing callus was observed at the end of 45 day.

Culture media and growth conditions for shoot induction

The callus formation continued for two months and then they were subcultured to MS basal media in jam bottles (500ml) with auxins reduced to 0.1mg/l and cytokinin concentration unchanged (Table 1) and transferred to culture room with light provided by fluorescent bulbs (Philips) with intensity of 2000-3000 lux and maintaining a photoperiod of 16 h per day with temperature of 25 ± 2°C.

A total of 27 explants with callus were maintained as three replications of nine explants each. Calli were cultured in jam bottles at the rate of one explant with callus per bottle. The media details are provided in Table 1.

After four weeks, the number of shoots that emerged from each combination was counted. These were again subcultured to MS basal media for rooting and maintained for 30-40 days. Analysis of variance was performed for the effect of plant growth regulators on callus induction and shoot induction separately. Means were compared using least significant difference test (LSD: $P<0.05$).

Experiments with petal explants

M5 media (MS basal with 2,4-D, (1 mg L^{-1}) and NAA (1 mg L^{-1}) along with 6-benzylaminopurine(BAP) at 0.5 mg L^{-1}) was prepared and used for culturing petal explants collected from the same mother plant. Surface sterilisation and culture conditions were the same as used for leaf explants. Thirty explants of 1-2mm sections were inoculated as three replications of ten each. These explants were further sub-cultured onto the MS basal media with 2,4-D, (0.1 mg L^{-1}) and NAA (0.1 mg L^{-1}) along with BAP at 0.5 mg $L^{-1}$and maintained for 1-2 months. Well-developed single embryos were transferred to MS basal media with BAP (0.5 mg L^{-1}) for plantlet formation and allowed to remain in the same media for a period of 30 days. The well-developed plants were then taken up for primary hardening. The details on the number of explants, number of somatic embryos and the number of plants regenerated are mentioned in Table 2.

Hardening

The well rooted plantlets were taken out from the culture media and washed with water carefully to remove the traces of agar and they were transplanted in jam bottles containing autoclaved mixture of Co-copit, Perlite and Vermicompost (2:1:1) for primary hardening and incubated in the same culture room in light at 25 ± 2°C. The plantlets (10-12 cm height) after sufficient growth were transferred from the bottles to plastic pots containing sterile mixture of sand, soil and vermicompost (1:2:1) and maintained in the polyhouse where in a temperature of 28±2°C with relative humidity of 65-75% was maintained for secondary hardening. The plantlets were irrigated with tap water twice in a week for a total period of two weeks to complete the acclimatization process. The plantlets obtained from leaf explants and petal explants were maintained separately.

DNA isolation and quantification

From two different batches of plants regenerated from leaf and petal explants, plantlets were selected for DNA extraction. Young leaves (300-400mg) were collected from selected plants and total genomic DNA was extracted using a cetyltrimethyl ammonium bromide (CTAB) procedure (Doyle and Doyle, 1990)[13]. 300-400mg of fresh leaves was ground to powder in liquid nitrogen using a mortar and pestle. The ground powder was transferred to a 50 ml falcon tube with 10 ml of CTAB buffer [2% (w/v) CTAB, 1.4 M NaCl, 20 mM Ethylene diamine tetraacetic acid, 100 mM Tris (tri (hydroxymethyl) amino methane]-HCl, and 0.2% (v/v) β-mercaptoethanol). The homogenate was incubated at 60^{o}C for 2 h, before extraction with an equal volume of chloroform/isoamyl alcohol (24:1 v/v) by centrifugation at 10,000 x *g* for 20 min. DNA was precipitated from the aqueous phase by mixing with an equal volume of isopropanol. After centrifugation at 10,000 x *g* for 10 min, the DNA pellet was washed with 70% (v/v) ethanol, air-dried and resuspended in TE (10 mM Tris-HCl, pH 8.0, and 0.1 mM EDTA) buffer. The quantification of isolated DNA was performed by visualizing under UV light, after electrophoresis on 0.8% (w/v) agarose gel at 60 V for 45 min and compared to a known amount of uncut lambda DNA (MBI, Fermentas). Quantified samples were then treated with 1µl RNase A per 100 µl DNA solution at 37 ^{o}C for one hour. DNA samples were finally diluted with sterile TE buffer to get the final concentration of 25 ng µl^{-1}. 2 µl of diluted DNA was subsequently used for polymerase chain reaction (PCR) amplification.

Primers

Twenty random primers (10-mer from Sigma-Aldrich Chemicals, St. Louis, USA) and twenty Universal ISSR primers (Integrated device technology, India), were used for initial DNA amplification and based on consistent results nine of each were selected for further analysis.

PCR Amplification conditions

Amplification with both RAPD (Williams *et al.,* 1990) and ISSR primers (Zietkiewicz *et al.,* 1994) was carried out in a total volume of 25 µl containing 2 µl (25 ng) of template DNA, 2.5 µl of 10X PCR buffer containing 15 mM MgCl2, 0.5 µl of dNTPs (10 mM each of dATP, dGTP, dTTP, dCTP), 1 µl of primer, 0.25 µl of *Taq* DNA polymerase and 18.75 µl of sterile MQ water.

Amplification conditions were performed as initial DNA denaturation at 95ºC for 4 min. followed by 35 cycles of 1 min denaturation at 95ºC, 1 min annealing at 37ºC and 2 min of extension at 72ºC with a final extension time at 72ºC for 10 min. In case of ISSR primers, optimal annealing temperature was adjusted according to the base concentrations of the primers. Hence, annealing temperature was optimized for each primer using a gradient PCR.

Electrophoresis of amplified DNA

Amplified DNA fragments were separated and visualized on a 1.2% (w/v) agarose gel stained with ethidium bromide 3 µl of 6X loading dye was added to 25 µl of amplified products and following homogenization, 5 µl of the resulting mixture was loaded onto a gel prepared in 0.5X TAE buffer. DNA ladder (1 Kb marker, #SM0313, Fermentas life science, Germany) was also loaded flanking the samples. The sizes of the amplicons were estimated by comparing with 1kb DNA ladder. The gel was visualized on a UV transilluminator and photographed by an Alphaimager Gel Documentation System (Alpha Innotech Corporation, USA).

Statistical data analysis

In both marker systems, RAPD and ISSR, scorable bands were recorded as present (1) or absent (0) and based on band data amplified. All the bands (Polymorphic and monomorphic) were taken into account for calculation of similarity with a view to avoid over/under estimation of the distance. Jaccard's coefficients of similarity (Jaccard, 1908) was measured and a dendrogram based on similarity coefficient generated by the Un-weight Pair Group method using arithmetic averages (UPGMA) (Sneath and Sokal, 1973), and Sequential Agglomerative Hierarchial Non-overlapping (SHAN) clustering. The statistical analysis was done using the software NTSYS-PC (Numerical Taxonomy System for Personal Computers, version 2.02i) (Rohlf, 1992).

Results and Discussion

In vitro responses from leaf explants

In leaf explants the response was seen as swelling and the initiation of callus was seen after 15-20 days on the cut edges of the leaf explant. By the end of 35-40 days the callus proliferated (Fig. 1A). The initiation of callus was influenced by the auxin concentration in the media. Though callus was produced in all combinations of plant growth regulators the maximum callus induction of 93% was seen in MS with 2,4-D, (1 mg L^{-1}) + NAA (1 mg L^{-1}) +Kin (0.5 mg L^{-1}) (M6) followed by 83% callus induction in MS with 2,4-D, (1 mg L^{-1}) + NAA (1 mg L^{-1}) +BAP (0.5 mg L^{-1}) (M5) which were not significantly different from each other. Callus induction percentage ranged from 26 to 93% in MS basal with six different combinations of plant growth regulators. When auxins 2,4-D and NAA were added at 2 mg L^{-1} singly in the media the callus induction was poor and it ranged from 26 to 52%. Cytokinins viz., kinetin and BAP did not have any significant effect on callus induction. Conversely auxins added singly and in combination had a significant difference in callus induction (Fig. 2). This kind of additive effect of auxins has been reported in other crops like sorghum (Liu *et al.,* 2013), potato (Kumlay, 2014). In *Chrysanthemum*, all the previous studies (Lema-Rumniska and Niedojadlo, 2014; Mandal and Datta, 2005; Naing *et al.,* 2013) have reported that single auxins in combination with cytokinins are capable of inducing callus and somatic embryogenesis but the effect of combination of auxins have not been reported so far. These calli were transferred to fresh media with auxins reduced (Table 1) for shoot multiplication. Shoot formation was seen within a month (Fig. 1B) from the calli. The calli derived from MS with 2,4-D produced more shoots with BAP than with Kinetin (Fig. 3). Calli derived from NAA showed less number of shoots both with BAP and kinetin. Shoot multiplication from the callus derived from MS with a combination of auxins was high and they were significantly different from other media. A maximum of 24 shoots were produced with callus derived in M5 media and this was significantly different from M6 media with Kinetin.

The least number of shoots was produced in M2 media (Fig. 3). When we compare all the combinations it is found that MS media with BAP was found to be better in induction of shoots than kinetin. This kind of higher response with BAP has been reported in *Chrysanthemum* (Naing *et al.,* 2013) and also in other crops like Banana (Muhammad *et al.,* 2007)and pigeon pea (Geetha *et al.,* 1998). This is attributed to the differences in the uptake rates reported in different genomes, varied translocation rates to meristematic regions, and metabolic processes in which cytokinin may be degraded or conjugated with sugars or amino acids to form

biologically inert compounds (Blakesly, 1991; Kaminek, 2002).

The shoots production continued till the end of second subculture and resulted in further shoot induction. At the end of three cycles it was found that a large number of plants (70-80 plants) could be regenerated from a single piece of leaf. The pathway of regeneration from leaf was indirect i.e. via callus. Single plantlets of 5-6cm height were transferred for rooting to MS basal media. 90-95% root formation was found within 10 -15 days and after 20-25 days they were removed for hardening.

In vitro responses from petal explants

In our experiments with petal explants, 10 explants were inoculated as three replications in M5 media and there was 100% response on this media for direct embryogenesis. There was profuse direct embryo formation from the surface within two weeks of culture (Fig. 1C). At the end of one month when they were observed under the stereo microscope the clear globular embryos with suspensor region was seen (Fig. 1D). An average of 54 distinct somatic embryos was observed from a single explant. These bipolar somatic embryos with root and shoot pole (Fig. 1E) were separated singly and on subculture to fresh MS basal media in light, regenerated into plantlets (Fig 1G). From a single petal explant we were able to obtain 18-20 plantlets (Table 2).

Hardening of *in vitro* regenerated plants

After sufficient root growth (Fig. 1H) the plantlets were removed for hardening The well rooted plantlets were taken out from the culture and washed with water carefully to remove the traces of agar and they were transplanted in jam bottles containing autoclaved mixture of Co-copit, Perlite and Vermicompost (2:1:1) for primary hardening and incubated in the same culture conditions for a month (Fig. 1F). After a month the well grown plantlets (Fig. 1I) from the bottles were transferred to pots containing sterile mixture of sand, soil and vermicompost (1:2:1) and they were transferred to the polyhouse where in a temperature of $28\pm2^{o}C$ with relative humidity of 65-75% was maintained for secondary hardening. The plantlets were irrigated with tap water twice in a week for a total period of two weeks to complete the acclimatization process. There was 90-98% survival of plants. These plants grew well and flowered (Fig. 1J).

Among the two explants studied, the response from petals is seen within 10-14 days and it is able to produce direct embryos and this may be due to the better meristematic nature of the petals. This kind of quick response and meristematic nature of petals of *Chrysanthemum* have been reported previously (Tanaka *et al.,* 2000; Mandal and Datta, 2005; Song *et al.,* 2011). The effect of growth plant growth regulators on adventitious shoot regeneration from leaf, stem, petiole and petal explants of *Chrysanthemum* was studied and it was reported that petals are the most responsive for shoot regeneration (Song *et al.,* 2011). A high concentration of auxin IAA along with BAP and Kinetin was required for shoot regeneration from different explants (Song *et al.,* 2011).

In *Chrysanthemum* a high genotypic influence has been reported by many researchers. For example in a study of somatic embryogenesis from 10 cultivars of the Lady group it is reported that the highest response for somatic embryogenesis was in Lady Salmon and in Lady yellow (Lema-Rumniska and Niedojadlo, 2014) when the 2,4-D was at a concentration of $4mgl^{-1}$. Direct embryogenesis from ray florets of five cultivars of *Chrysanthemum* with a maximum

response was 40% with the production of 7 embryos per explants was reported (Mandal and Datta, 2005). In our current protocol the advantage is that we are able to obtain somatic embryogenesis with low concentrations of auxins and also obtained higher percentage embryogenesis and somatic embryos from the cultivar Pusa centenary which proves its amenability for large scale culture.

Genetic fidelity analysis of *in vitro* regenerated plants

The 9 RAPD primers produced 28 distinct and scorable bands in the size range of 300 bp (OPG-08) to 2000 bp (OPD-05). The number of scorable bands for each primer varied from 1 (OPD-20) to 7 (OPG-08) with an average of 3.11 bands per primer (Table 3). These primers did not show any polymorphism between them and the mother plants. PIC value of all RAPD primers have zero and morphologically all the clones (L1 to L10 were leaf regenerated and P1 to P20 were petal regenerated) were similar to the mother plant, indicating no variations. A representative pattern of amplification obtained with primers OPG 7 and OPG 11 are provided in Figure 4A and 4B. The ISSR primers used in the present study were mostly dinucleotide repeats with one tetranucleotide repeats. Of the 10 primers tested, 9 contained 3(GA)n, 1(CA)n, 3(TC)n and 2(AG)n and one 5'-tetraanchored primers. ISSR primers produced 47 distinct and scorable bands in the size range of 300 bp (ISSR-27) to 2000 bp (ISSR-8).

The number of scorable bands for each primer varied from 1 (ISSR-21) to 9 (ISSR-12) with an average of 4.7 bands per primer (Table 4). Calculated PIC value varied from 0.0 (ISSR-7) to 0.489 (ISSR-2) and average PIC value per primer was 0.048. Out of 10 ISSR primers, 9 primers showed monomorphic banding pattern in both leaf and petal derived plants and the mother plant. However, only one polymorphic band was detected with ISSR-2 in one of the leaf derived plantlets L1. A unique band of 1500 bp was present in L1, which was absent in mother plant as well as in other *in vitro* raised plantlets (Fig. 4E). The polymorphism in tissue cultured raised plants could result from change in either the sequence of primer binding site (e.g. point mutations) or change which alter the size and prevent successful amplification of target DNA (e.g. insertion, deletion, inversions) (e.g. point mutations) or change which alter the size and prevent successful amplification of target DNA (e.g. insertion, deletion, inversions) or alteration in DNA methylation during *in vitro* culture (Saker *et al.,* 2000).

The PCR amplification pattern obtained with primers ISSR 7, ISSR12 and ISSR 2 is represented in Figure 4C, 4D and 4E. The use of molecular markers for studying the genetic stability has been emphasized by many workers (Jayanthi and Mandal, 2001; Bhatia *et al.*, 2011; Saha *et al.*, 2014).

The genetic stability of the clones depends on the genotype (Smith, 2008), cultural conditions, maintenance of cultures (Haisel *et al.*, 2001) and number of sub cultural passages (Chaterjee and Prakash, 1996). In this study it was found that callus derived plantlets are showing some amount of variation while the embryo derived ones are completely uniform.

The superiority of somatic embryogenesis derived plantlets has been confirmed by many studies and this may be due to the fact that the developmental constraints required by somatic embryos exert selection against variant ones. Using two marker systems for evaluation of somaclonal variation has been always advantageous (Palombi and Damiano, 2002).

Table.1 Details of Media used for callus induction and shoot multiplication from leaf explants in chrysanthemum

Media code	Callus induction media				Shoot multiplication media			
	(mg L^{-1})							
	2,4-D	**NAA**	**BAP**	**KIN**	**2,4-D**	**NAA**	**BAP**	**KIN**
M1	2	0	0.5	0	0.1	0	0.5	0
M2	2	0	0	0.5	0.1	0	0	0.5
M3	0	2	0.5	0	0	0.1	0.5	0
M4	0	2	0	0.5	0	0.1	0	0.5
M5	1	1	0.5	0	0.05	0.05	0.5	0
M6	1	1	0	0.5	0.05	0.05	0	0.5

Table.2 Details of experiment with petals as explants in MS media

Total No. of explants	% embryogenesis	No. of somatic embryos	No of plants regenerated	No. of plants hardened
30 (10 explants as three replications)	100*	54*	20*	18.33*

*Average of three replications

Table.3 Details of RAPD primers, sequences, number and size of amplified fragments obtained with *in vitro* regenerated plants of chrysanthemum

S.No	Primers	5'–3' sequence	Tm (^{o}C)	No. of bands per primer	Size range (bp)
1	OPD-05	TGAGCGGACA	37.1	3	700-2000
2	OPD-07	TTGGCACGGG	40.9	3	700-1900
3	OPD-20	ACCCGGTCAC	39.1	1	~650
4	OPF-10	GGAAGCTTGG	32.3	1	~500
5	OPF-11	TTGGTACCCC	32.6	4	450-1500
6	OPG-05	CTGAGACGGA	32.8	2	1000-1300
7	OPG-08	TCACGTCCAC	34.5	7	300-1500
8	OPG-11	TGCCCGTCGT	43.2	2	450-750
9	OPH-05	AGTCGTCCCC	38	5	850-1700

Table.4 Details of ISSR primers and amplification products obtained with *in vitro* regenerated plants of chrysanthemum

S.No	Primers	5' to 3' motif	Tm (^{o}C)	No. of bands per primer	Total bands	Size range of bands(bp)
1	ISSR-12	$(GA)_7A$	45.7	9	189	300-1400
2	ISSR-14	$(CT)_7A$	44.7	5	105	500-2000
3	ISSR-16	$(CA)_7G$	51	4	84	550-1500
4	ISSR-2	$(TC)_7A$	47	4	84	500-1500
5	ISSR-21	$(TC)_7C$	48.1	1	21	900
6	ISSR-27	$(ATAG)_4$	39.6	6	126	300-1500
7	ISSR-7	$(AG)_7T$	47	3	63	500-1100
8	ISSR-10	$(GA)_7T$	45.4	5	105	500-1500
9	ISSR-8	$(AG)_7C$	48.8	6	126	300-2000
10	ISSR-11	$(GA)_7C$	46.8	4	84	600-1400

Fig.1 A–E Plant regeneration via embryogenesis from leaf and petals of *Chrysanthemum*. A. Embryogenic callus induction from leaf explants after one month of incubation in dark. B. Plantlet formation from embryogenic calli of leaf explants. C. Direct embryogenesis from petal explants. D. Distinct globular somatic embryos observed from petal explants. E. Single somatic embryo with root and shoot pole. F. Rooted plantlets in primary hardening media. G. Plantlet formation from somatic embryo. H. Rooting of *in vitro* derived plantlets in MS basal media. I. Hardened plants ready for secondary hardening in mist chamber. J. Completely hardened plants flowering in mist chamber

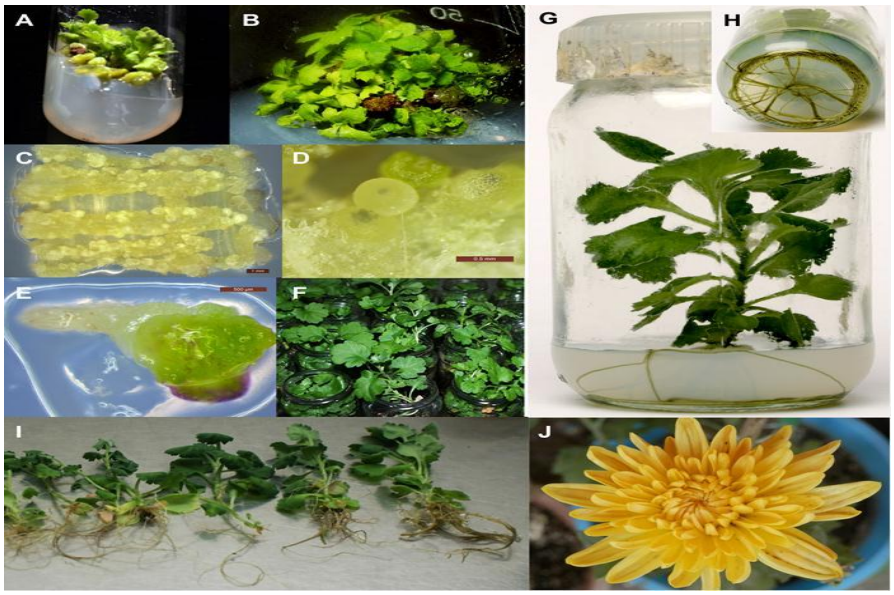


Fig.2 Callus induction obtained with different media (details of media in Table 1) using leaf explants of Pusa centenary. Values are Mean ± standard error. Means followed by same letter are not significantly different at p≤0.05

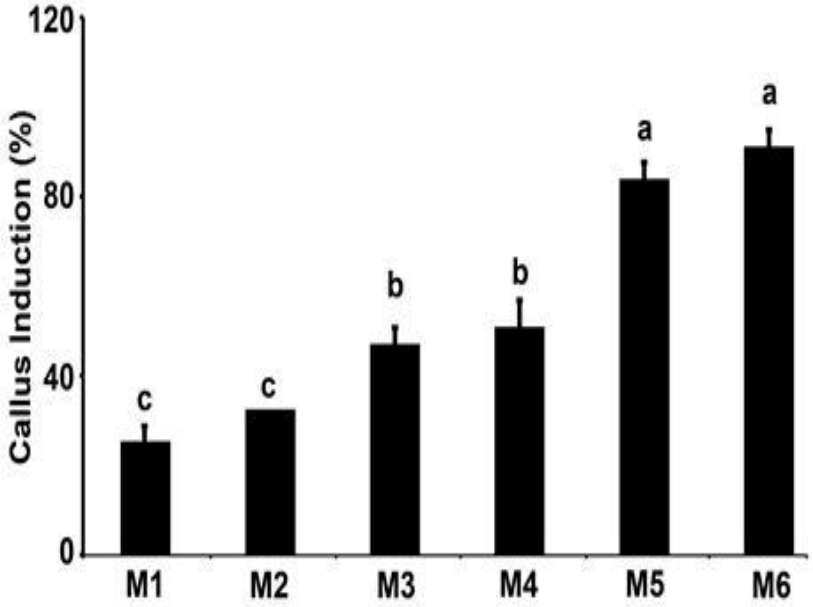


Fig.3 Number of shoots obtained with different media (details of media in Table 1) in Pusa centenary. Values are Mean ± standard errors. Means followed by same letter are not significantly different at p≤0.05

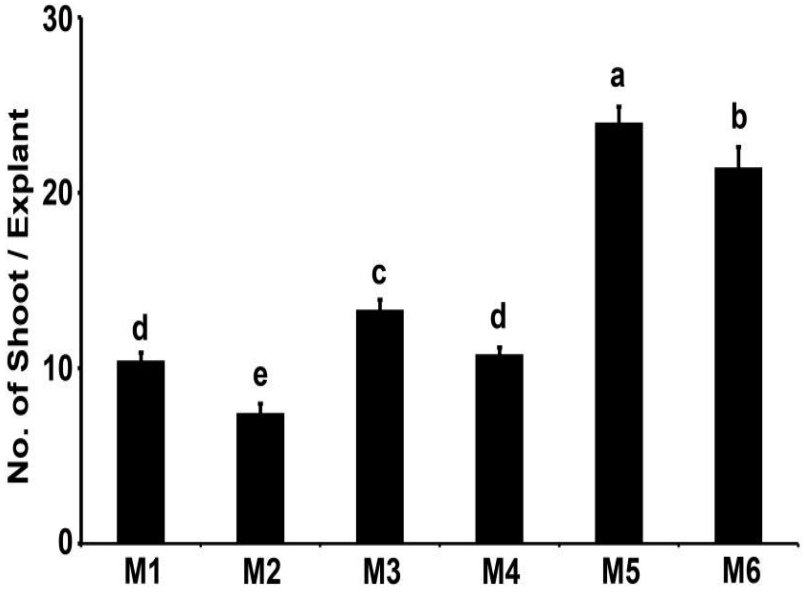
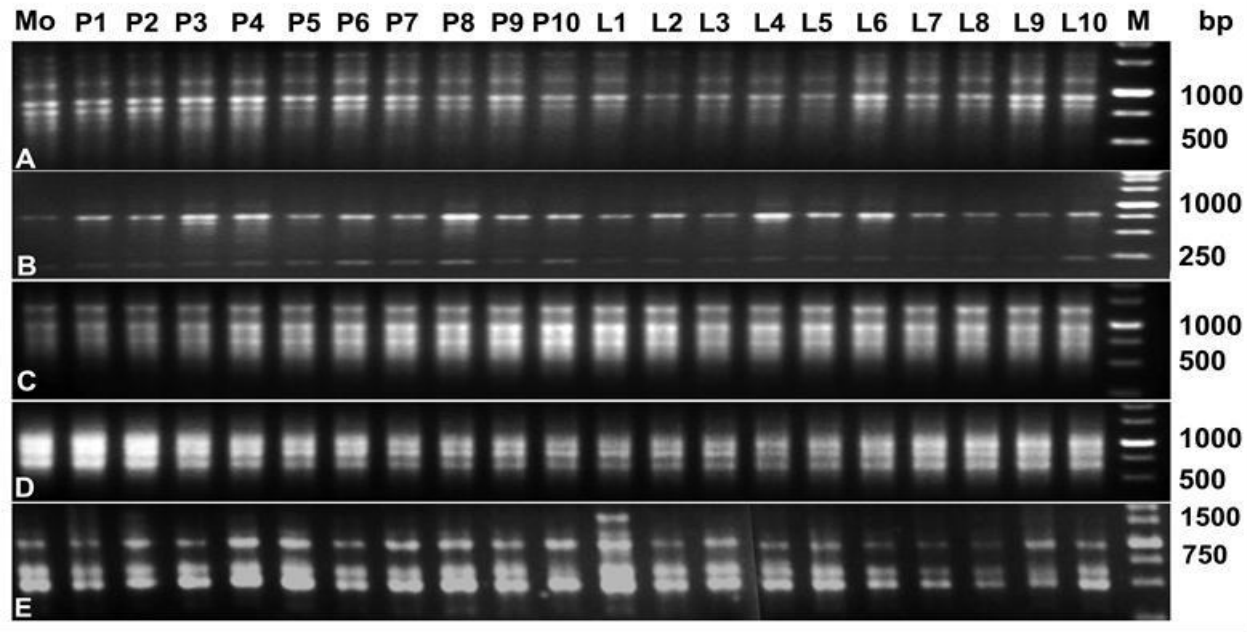


Fig.4 A and 4B: RAPD banding pattern in micropropagated plants from leaf and petal explants compared with mother plant of Pusa centenary using primers OPG 11 and OPD 7. (Mo- mother plant; P1 to P10 micropropagated plants from petal explants and L1 to L10 micropropagated plants from leaf explants; M 100 bp marker).
Fig.4C, 4D and 4E: ISSR banding pattern in micropropagated plants from leaf and petal explants compared with mother plant of Pusa centenary obtained with ISSR 7, ISSR 12 and ISSR 2(Mo- mother plant; P1 to P10 micropropagated plants from petal explants and L1 to L10 micropropagated plants from leaf explants; M 100 bp marker)



It has been assumed that the use of two markers, which amplify different regions of the genome, allows for better chances for the identification of genetic variations in the clones. These two marker systems viz., RAPD and ISSR have been extensively used to detect somaclonal variation in many other crops. ISSR markers are able to bring out the genomic differences than RAPD as is seen in our studies. ISSR always reveals more polymorphism than RAPD as is reported in case of coffee, tea and banana (Rani and Raina, 2000; Devarumath *et al.*, 2002; Ray *et al.*, 2006).

The ISSRs are widely distributed throughout the genomic DNA and make amplification of genomic DNA possible in much larger numbers of fragments per primer. This higher amplification efficiency of ISSRs makes the genetic inferences much clearer with some clues for genetic variation at inter- and even intra-specific levels (Powell *et al.*, 1996). Our study proves that if true to type plants are required direct embryogenesis is the preferred method. Along with several other advantages of direct somatic embryogenesis, this protocol opens up the prospect of genetic transformation and bioreactor based propagation in this important commercial cultivar.

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References

Bhatia, R., Singh, K. P., Sharma, T. P., Jhang, T., 2011.Evaluation of the genetic fidelity of *in vitro*-propagated gerbera *(Gerbera jamesonii* Bolus) using DNA-based markers. *Plant cell tissue organ cult.*, 104(1): 131–135.

Blakesly, D.,1991. Uptake and metabolism of 6-benzyladenine in shoot cultures of *Musa* and *Rhododendron*. *Plant Cell, Tissue Organ Cult.*, 25 (1): 69-74.

Chaterjee, G. and Prakash, J., 1996. Genetic stability in commercial tissue culture, In: Plant Biotechnology: Commercial Prospects and Problems (Eds.) J Prakash and RIM Pierik. Oxford IBH Publishing Co. New Delhi, India, Pp. 111–121.

Devarumath, R. M., Nandy, S., Rani, V., Marimuthu, S., Muraleedharan, N., 2002. RAPD, ISSR and RFLP finger printing as useful markers to evaluate genetic integrity of micropropagated plants of three diploid and triploid elite tea clones representing *Camellia sinensis* (China type) and *C. assamica* ssp. *assamica* (Assam-India type). *Plant Cell Rep*, 21(2): 166-173.

Doyle, J. J., Doyle, J. L., 1990. Isolation of plant DNA from fresh tissue. *Focus*, 12(1): 13-15.

Geetha, N., Venkatachalam, P., Prakash, V., Lakshmi, S. G., 1998. High frequency induction of multiple shoots and plant regeneration from seedling explants of pigeon pea (*Cajanus cajan* L.). *Curr. Sci.*, 75 (10): 1036–1041.

Gould, A. R., 1986. Factors controlling generation of variability in vitro, In: Cell Culture and Somatic Cell Genetics in Plants (Eds.) Vasil, I. K., Vol 3. Plant Regeneration and Genetic Variability. Academic Press Inc. New York. Pp. 549-567.

Haisel, D., Hofman, P., Vagneri, M., Lipavska, H., Ticha, L., Schafer, C., Capkova, V., 2001. *Ex vitro* phenotype stability is affected by *in vitro* cultivation. *Biol. Plant*, 44(3): 321-324.

Himstedt, J. P., Jacobsen, H. J., Fisher-Kluver, G., 2001. Shoot regeneration from stem and leaf explants of *Chrysanthemum* (*Dendranthema x grandiflorum*), *Acta Hortic.*, 560: 421–424.

Jaccard, P., 1908. *Nouvellesrecherchessurla* distribution florale, *Bull. Soc. Vaud. Sci. Nat*, 44: 223–270.

Jayanthi, M., and Mandal, P. K., 2001. Plant regeneration through somatic embryogenesis and rapd analysis of regenerated plants in *Tylophora indica* (Burm. F. Merrill.). *In vitro Cellular and Development Biology*, 37 (5): 576-580.

Kaminek, M., 2000. Progress in cytokinin research. *Trends Biotechnol.*, 10: 159-162.

Kumlay, A. M., 2014. Combination of the Auxins NAA, IBA, and IAA with GA_3 Improves the Commercial Seed-Tuber Production of Potato (*Solanum tuberosum* L.) under *In vitro* Conditions. *BioMed Research International*, 14: 439259.

Larkin, P., and Scowcroft, W. R., 1981. Somaclonal variation, a novel source of variability from cell cultures for plant improvement, *Theor. Appl. Genet.* 60 (4): 197–214.

Lema-Rumniska, J., and Niedojadlo, J., 2014.Somatic embryogenesis in leaf explants of *Chrysanthemum radiomutants* of the 'lady' group. *Propagation of ornamental plants*, 14 (4): 177-183.

Liu, G., Gilding, E. K., Godwin, I. D., 2013. Additive effects of three auxins and copper on sorghum in vitro root induction. *In Vitro Cell. Dev. Biol.*-

Plant, 49 (2): 191-197.

Mandal, A. K. A., Datta, S. K., 2005. Direct somatic embryogenesis and plant regeneration from ray florets of *Chrysanthemum*, *Biologia Plantarum*, 49 (1): 29-33.

Muhammad, A., Rashid, H., Hussain, I., Saqlan Naqvi, S. M., 2007. Proliferation-rate effects of BAP and Kinetin on Banana (*Musa* spp. AAA Group) 'Basrai'. *Hortscience*, 42(5): 1253-1255.

Murashige, T., Skoog, F., 1962.A revised medium for rapid growth and bioassays with tobacco tissue cultures.*Physiol. Plant,* 15: 473-97.

Naing, A. H., Min, J. S., Park, K., Chung, M. Y., Lim, S. H., Lim, K. B., Kil, K. C., 2013. Primary and secondary somatic embryogenesis in *Chrysanthemum* (*Chrysanthemum morifolium*) cv. 'Baeksun' and assessment of ploidy stability of somatic embryogenesis process by flow cytometry. *Acta Physiol Plant*, 35: 2965–2974.

Palombi, M., and Damiano, C., 2002.Comparison between RAPD and SSR molecular markers in detecting genetic variations in kiwifruit (*Actinida deliciosa* A. chev.).*Plant Cell Rep,* 20(11): 1061-1066.

Petty, L. M., Harberd, N. P., Carre´, I. A., Thomas, B., Jackson, S. D., 2003. Expression of the *Arabidopsis* gai gene under its own promoter causes a reduction in plant height in *Chrysanthemum* by attenuation of the gibberellin response. *Plant Sci.*, 164 (2): 75–182.

Powell, W., Machray, G. C., Provan, J., 1996. Polymorphism revealed by simple sequence repeats. *Trends in Plant Science*, 1(7): 215-222.

Pradeep, R., Sarla, N., Siddiq, E. A., 2002. Inter simple sequence repeats (ISSR) polymorphism and its application in plant breeding. *Euphytica*, 128 (1):9-17.

Ram, R., Verma, N., Singh, A. K., Zaidi, A. A., 2009. Virus-free *Chrysanthemum*: Production and quality management. *Arch. Phytopathol. Plant Prot.*, 42(10): 940-949.

Rani, V., and Raina, S. N., 2000. Genetic fidelity of organized meristem-derived micropropagated plants: a critical reappraisal. *In Vitro Cell Dev. Biol. Plant*, 36(5): 319-330.

Ray, T., Dutta, I., Saha, P., Das, S., Roy, S. C., 2006.Genetic stability of three economically important micropropagated banana (Musa spp.) cultivars of lower Indo-Gangetic plains, as assessed by RAPD and ISSR markers. *Plant Cell, Tissue and Organ Cult.*, 85 (1): 11-21.

Rohlf, F. J., 1992. NTSYS-PC: Numerical taxonomy and Multivariate Analysis System. Exeter Software, New York.

Saha, S., Roy, S., Sengupta, C., Ghosh, P. D., 2014. Micropropagation and analysis of genetic stability in regenerated plantlets of *Ocimumcanum* Sims. *Indian J. Plant Physiol.*, 19 (2): 174–183.

Saker, M. M., Bekheet, S. A., Taha, H. S., Fahmy, A. S., Moursy, H. A., 2000. Detection of somaclonal variations in tissue cultured-derived date palm plants using isoenzyme analysis and RAPD fingerprints. *Biol. Plant,* 43 (3): 347-351.

Smith, M. K., 2008. A review of factors influencing the genetic stability of micropropagated bananas. *Fruits*, 43: 219–223.

Sneath, P. H. A., and Sokal, R. R., 1973. Numerical taxonomy: the principles and practice of numerical classification. WH Freeman, San Francisco. Pp. 573

Song, J. Y., Mattson, N. S., Jeong, B. R.,

2011. Efficiency of shoot regeneration from leaf, stem, petiole and petal explants of six cultivars of *Chrysanthemum morifolium. Plant Cell, Tissue Organ Cult.*, 107: 295-304.

Tanaka, K., Kanno, Y., Kudo, S., Suzuki, M., 2000. Somatic embryogenesis and plant regeneration in *Chrysanthemum* (*Dendranthem agrandiflorum* (Ramat.) Kitamura). *Plant Cell Reports*, 19(10): 946-953.

Teixeira da Silva, J. A., 2004. Ornamental *Chrysanthemums*: improvement by biotechnology. *Plant Cell, Tissue Organ Cult.*, 79(1): 1-18.

Waseem, K., Jilani, M. S., Khan, M. S., 2009. Rapid plant regeneration of *Chrysanthemum* (*Chrysanthemum morifolium* L.) through shoot tip culture. *Afr. J. Biotechnol.*, 8(9):1871–1877.

Williams, J. G. K., Kubelik, A. R., Livak, K. J., Rafalski, J. A., Tingey, S. V., 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Res.*, 18(22): 6533-6535.

Zietkiewicz, E., Rafalski, A., Labuda, D., 1994. Genomic fingerprinting by simple sequence repeat (SSR)-anchored polymerase chain reaction amplification, *Genomics*, 20 (2): 176-183.

Zilberman, D., and Henikoff, S., 2007. Genome-wide analysis of DNA methylation patterns, *Development.* 134(22): 3959-3965.

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