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Morphological and Molecular Based Genetic Stability Assessment of *in Vitro* Propropagated Potato Micro-plants.

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Abstract: *Genetic stability of in vitro propagated plants for tissue-culture-based seed potato production was assessed using molecular markers and morphological traits. In vitro propagated potato mother plant and its derivatives (10th and 20th cycle's regenerants) sub-cultured on two MS media (with and without growth regulators) were used in analysis. In the DNA analysis, a total of 38 (10 RAPD, 9 ISSR and 5 SSR) primers produced a total of 152 (53 RAPD, 56 ISSR and 43 SSR) clear, distinct and reproducible amplicons. All the banding profiles from micro-propagated regenerants were similar to the mother plant. Cluster analysis revealed 100% genetic similarity among the mother plant and its derived regenerants within the corresponding cluster (I-IX). Principal component analysis (PCA) also plotted mother plant and its genotypes of each cluster together. In the morphological assessment, significant genotypic effect was observed in the different phenotypic traits, however, there was no significant difference between 10th and 20th cycle regenerants. Moreover, there were no molecular as well as morphological differences observed by the additional supplementation of growth regulators in the MS medium. This study confirmed that the true-to-type nature of the in vitro propagated plants can be multiplied upto 20 cycles of regeneration and is a safe method for rapid seed production through tissue culture.*

Keywords: *Genetic stability, in vitro propagation, molecular markers, seed potato, Solanum tuberosum.*

1. INTRODUCTION

Tissue culture technologies have been proven very valuable in potato for maintenance of healthy stocks and rapid propagation of planting materials (Smulders and Klerk, 26). In general, clonal propagation through tissue culture should generate individuals identical to the mother plant from which they were sub-cultured. In India, traditionally, seed potato is produced by repeated clonal multiplications of initial disease-free tubers and progressive accumulation of degenerative viral diseases results low yield (Naik *et al.,* 18). One of the major causes of low Indian potato productivity (19.9 tonnes/ha) (NHB, 19) is use of poor quality seed (Singh, 26), as degenerated seed is known to lower productivity (Kumar *et al.*, 10). At present the state and central seed production agencies of the country are able to meet only 20-25% requirement of quality seed (Kumar *et al.*, 11). For bridging this wide gap, large-scale integration of conventional and innovative methods like micro-propagation at commercial level is needed for producing enough quantity of healthy seed tubers in minimum period of time (Pandey, 20). *In vitro* produced disease-free potato clones combined with conventional multiplication methods have become an integral part of seed production in many countries including India (Naik *et al.*, 18).

Moreover, Tsoka *et al.* (29) demonstrated potato seed tuber production from *in vitro* and apical stem cutting under aeroponic system. Kumar *et al.* (10) reported that more than 70% *in vitro* plantlets were survived in direct transplanting and seed potato crop can be raised for minituber production under

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net house conditions. As per the minimum certification standards the materials initiated for mass should be multiplied only up to 8-12 cycles under *in vitro* condition. However, in this context no scientific study has been carried out in potato on effect of clonal regeneration by sub-culturing at genetic level.

The past studies have shown that *in vitro* cultures pose a problem of genetic stability caused by genetic and epigenetic variations (somaclonal variation) in regenerants. This may lead to phenotypic changes in the propagated clones and alternation of in the agronomic performance (Vazquez and Linacero, 31). So in the clonal regeneration, one of the most crucial concerns of curators is to retain genetic stability of *in vitro* propagating material. Hence a quality check up for true to type planting material at an early stage of development is considered to be very useful in plant tissue culture (Zilberman *et al*., 34).

Molecular markers serve as an important tool to check the genetic uniformity and true to type nature of micro-propagated plants. In the past RAPD and ISSR markers have been proven to be quite efficient in detecting genetic fidelity in several plant species such as *Solanum* species (Zarghami *et al*., 33; Dann and Wilson, 4; Aversano *et al*., 2), *Gynura bicolor* (Liu *et al*., 14), *Malus domestica* (Pathak and Dhawan, 21), *Simmondsia chinensis* (Kumar *et al*., 12) and *Lilium orientalis* (Liu and Yang, 15). The aim of the present study was to assess the genetic stability of *in vitro* propagated plants using molecular markers (RAPD, ISSR and SSR) and morphological characters for tissue-culture-based rapid seed potato production.

Table 1
Potato varieties used to assess in genetic stability using molecular markers

SN[a]	Sample	Code[b]	SN[a]	Sample	Code[b]
1.	Kufri Bahar-Mother plant	KBR1	25.	Kufri Himalini GR-20th cycle	KHN5
2.	Kufri Bahar WGR-10th cycle	KBR2	26.	Kufri Lauvkar-Mother plant	KLR1
3.	Kufri Bahar GR-10th cycle	KBR3	27.	Kufri Lauvkar WGR-10th cycle	KLR2
4.	Kufri Bahar WGR-20th cycle	KBR4	28.	Kufri Lauvkar GR-10th cycle	KLR3
5.	Kufri Bahar GR-20th cycle	KBR5	29.	Kufri Lauvkar WGR-20th cycle	KLR4
6.	Kufri Chandramukhi-Mother plant	KCM1	30.	Kufri Lauvkar GR-20th cycle	KLR5
7.	Kufri Chandramukhi WGR-10th cycle	KCM2	31.	Kufri Pukhraj-Mother plant	KPJ1
8.	Kufri Chandramukhi GR-10th cycle	KCM3	32.	Kufri Pukhraj WGR-10th cycle	KPJ2
9.	Kufri Chandramukhi WGR-20th cycle	KCM4	33.	Kufri Pukhraj GR-10th cycle	KPJ3
10.	Kufri Chandramukhi GR-20th cycle	KCM5	34.	Kufri Pukhraj WGR-20th cycle	KPJ4
11.	Kufri Chipsona-3-Mother plant	KC31	35.	Kufri Pukhraj GR-20th cycle	KPJ5
12.	Kufri Chipsona-3 WGR-10th cycle	KC32	36.	Kufri Sindhuri-Mother plant	KSN1
13.	Kufri Chipsona-3 GR-10th cycle	KC33	37.	Kufri Sindhuri WGR-10th cycle	KSN2
14.	Kufri Chipsona-3 WGR-20th cycle	KC34	38.	Kufri Sindhuri GR-10th cycle	KSN3
15.	Kufri Chipsona-3 GR-20th cycle	KC35	39.	Kufri Sindhuri WGR-20th cycle	KSN4
16.	Kufri Girdhari-Mother plant	KGR1	40.	Kufri Sindhuri GR-20th cycle	KSN5
17.	Kufri Girdhari WGR-10th cycle	KGR2	41.	Kufri Surya-Mother plant	KSY1
18.	Kufri Girdhari GR-10th cycle	KGR3	42.	Kufri Surya WGR-10th cycle	KSY2
19.	Kufri Girdhari WGR-20th cycle	KGR4	43.	Kufri Surya GR-10th cycle	KSY3
20.	Kufri Girdhari GR-20th cycle	KGR5	44.	Kufri Surya WGR-20th cycle	KSY4
21.	Kufri Himalini-Mother plant	KHN1	45.	Kufri Surya GR-20th cycle	KSY5
22.	Kufri Himalini WGR-10th cycle	KHN2			
23.	Kufri Himalini GR-10th cycle	KHN3			
24.	Kufri Himalini WGR-20th cycle	KHN4			

[a] SN was used to depict gel pictures of the genotype

[b] Code was used in result and discussion of the paper

2. MATERIALS AND METHODS

2.2 Plant Materials and Culture Conditions

In the present study, nine commercial Indian potato varieties namely Kufri Bahar, Kufri Chandramukhi, Kufri Chipsona-3, Kufri Girdhari, Kufri Himalini, Kufri Lauvkar, Kufri Pukhraj, Kufri Sindhuri and Kufri Surya were used to assess genetic fidelity for tissue-culture based seed production. *In vitro* plants were multiplied from the mother plant of each variety by three double node cuttings dissected essentially from middle portion of the micro-plants in a test tube (25 × 150 mm) on MS medium (Murashige and Skoog, 17) (pH 5.8) supplemented with 4.19 µM D-calcium pantothenate, 30 gl^{-1} sucrose and 7 gl^{-1} agar at 20°C under a 16-h photoperiod (light intensity 50-60 µmol m^{-2} s^{-1}) in the environment controlled chamber. Two MS media were used to culture each variety as below:

(i) ***MS medium with growth regulator*:** The MS medium supplemented with NAA 0.05 µM and GA_3 0.29 µM. The growth regulators were used in the medium to detect induced, if any, genetic changes.

(ii) ***MS medium without growth regulator:*** No growth regulator was added in the MS medium and this is the most commonly used protocol.

The *in vitro* mother plant, 10th and 20th cycles sub-cultured regenerants from the mother plant of each variety were used for the assessment of genetic fidelity (Table 1).

2.2 DNA Analysis

Plant DNA was isolated from 100 mg leaves collected from fresh *in vitro* sub-cultures using the GenElute Plant Genomic DNA MiniPrep Kit (Sigma-Aldrich, St. Louis, USA). DNA quality and quantity were determined with NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, USA), and quality was also assessed on 0.8% (w/v) agarose gel. The isolated DNA was used for RAPD, ISSR, SSR and AFLP analyses.

Random amplified polymorphic DNA (RAPD) analysis of the potato genotypes was performed using random decamer primers (Operon Biotechnologies GmbH, Cologne, Germany). RAPD primers used in the analysis are summarized in Table 2. The polymerase chain reaction (PCR) was performed in a Mastercycler Gradient (Eppendorf, Hamburg, Germany) in a total volume of 10 µl and consisted of 50 ng DNA templates in 1 × PCR buffer, 2.5 mM $MgCl_2$, 200 µM dNTP, 0.5 µM of primer, 1 Unit Taq Polymerase (Qiagen). The PCR procedure included: one cycle of 4 min at 94°C, 1 min at 36°C and 2 min at 72°C followed by 43 cycles of 1 min at 94°C, 1 min at 36°C, and 2 min at 72°C, with a final extension of 8 min at 72°C. The amplified DNA products were separated by electrophoresis on a 1.6% agarose gel staining with ethidium bromide (0.5 µg ml^{-1}) in 0.5 × TBE buffer (Tris-borate-EDTA) using horizontal gel electrophoresis system Sub-Cell GT (Bio-Rad, USA) at room temperature. The gels were visualized under Gel Doc System (Alpha Innotech, San Leandro, CA) and bands were scored using the AlphaImager 3400 software (Alpha Innotech) by comparing with 100 bp DNA ladder (Fermentas, Burlington, Canada).

Inter simple sequence repeat (ISSR) analysis was performed using genomic DNA by ISSR primers summarized in Table 2. The polymerase chain reaction (PCR) was performed in a Master cycler Gradient (Eppendorf, Hamburg, Germany) in a total volume of 10 µl and consisted of 50 ng DNA templates in 1 × PCR buffer, 2.5 mM $MgCl_2$, 200 µM dNTP, 0.5 µM of primer, 1 Unit Taq Polymerase (Qiagen). The PCR procedure included: 5 min at 94°C followed by 35 cycles of 1 min at 94°C, 1 min at 55°C, and 2 min at 72°C, with a final extension of 8 min at 72°C. Gel documentation and analysis was performed like RAPD analysis.

Simple sequence repeat (SSR) analysis was performed using genomic DNA by SSR primers summarized in Table 2. SSR analysis was carried out in a total volume of 10 µl containing 50 ng of genome DNA using Ampli Taq Gold ® PCR Master Mix that includes Ampli Taq Gold DNA Polymerase (0.05 U/µl) for automated Hot Start PCR, 1 × Gold PCR Buffer (30 mM Tris/HCl, pH 8.05, 100 mM KCl), 400 µM each dNTP, 5mM $MgCl_2$ and 0.5 µM each primer. The PCR profile was a hot start of 94°C for 5 min, followed by 30 cycles of 94°C for 1 min, [Tm] for 2 min, 72°C for 2 min, and 72°C for 30 min.

Table 2
RAPD, ISSR and SSR primers sequence and amplicons profiles of *in vitro* propagated potato cultivars

S. No.	Primers	Sequence 5¹~-3¹~	Band size range (bp)	No. of total bands	Size of each band (bp)
	RAPD				
1.	OPB01	GTTTCGCTCC	700-900	2	700, 900
2.	OPB02	TGATCCCTGG	500-920	5	500, 700, 750, 850, 920
3.	OPB03	CATCCCCCTG	300-800	5	300, 500, 600, 700, 800
4.	OPB04	GGACTGGAGT	225-2000	4	225, 450, 600, 2000
5.	OPB05	TGCGCCCTTC	400-1500	6	400, 600, 630, 700, 900, 1500
6.	OPB06	TGCTCTGCCC	250-900	5	250, 350, 500, 650, 900
7.	OPB07	GGTGACGCAG	200-1500	9	200, 300, 350, 420, 500, 650, 900, 1000, 1500
8.	OPB08	GTCCACACGG	600-2000	6	600, 700, 750, 900, 1000, 2000
9.	OPB09	TGGGGGACTC	600-1800	7	600, 700, 850, 900, 1200, 1500, 1800
10.	OPB10	CTGCTGGGAC	300-700	4	300, 500, 600, 700
ISSR					
1.	ISSR5	BDB(TCC)5	200-600	4	200, 400, 500, 600
2.	ISSR19	(GCC)5	320-1300	7	320, 500, 550, 650, 750, 800, 1300
3.	ISSR1425	BDV(CAG)5	300-1000	5	300, 600, 700, 800, 1000
4.	ISSR2103	HVH(GTC)5	200-3000	10	200, 400, 500, 600, 650, 700, 1200, 1500, 2000, 3000
5.	ISSR2105	BDB(GAC)5	250-1500	5	250, 400, 500, 700, 1500
6.	ISSRMC1	VHV(GT)7G	200-1000	7	200, 250, 300, 400, 700, 800, 1000
7.	ISSRP92	BDB(CA)7	300-1500	5	300, 500, 700, 1200, 1500
8.	ISSRP93	BDB(CAC)5	300-1500	8	300, 400, 450, 520, 650, 800, 1200, 1500
9.	ISSRAm2	(CAG)5	300-1000	5	300, 500, 600, 700, 1000
	SSR				
1.	STIKA	F: TTCGTTGCTTACCTACTA R: CCCAAGATTACCACATTC	138-200	9	138, 143, 148, 154, 170, 176, 180, 190, 200
2.	STU6	F: GAAGTTTTATCAGAATCC R: ATCACCTCATCAGCAATC	157-230	8	175, 190, 195, 200, 213, 220, 225, 230
3.	STM5127	F: TTCAAGAATAGGCAAAACCA R: CTTTTTCTGACTGAGTTGCCTC	161-272	10	161, 166, 178, 216, 231, 237, 241, 250, 268, 272
4.	STM5114	F: AATGGCTCTCTCTGTATGCT R: GCTGTCCCAACTATCTTTGA	232-304	10	232, 257, 265, 275, 279, 284, 287, 293, 296, 304
5.	STM1053	F: TCTCCCCATCTTAATGTTTC R: CAACACAGCATACAGATCATC	118-170	6	118, 123, 130, 146, 165, 170

Tm of the primers were followed as described by Ghislain *et al.* (6). The 3 µl of selectively amplified sample was mixed with 0.3 µl of ROX labeled internal size standard GeneScan-500 ROX (PE Applied Biosystems), 12 µl deionized Hidi formamide denatured for 5-min at 95°C and quickly chilled on ice followed by 15 µl of the mixture was loaded into each sample tube and analysed on '3500 Genetic Analyzer' (Applied Biosystems, California, USA). Fragment analysis of SSR data was performed using GeneMapper ® Software Version 4.1 (Applied Biosystems). A 500-bp 'GS 500 ROX' standard was used to estimate the molecular size of the fragments. Only clear and reproducible peaks that appeared in independent PCR amplifications were scored.

2.3 Scoring and the Data Analysis

All reactions were repeated at least twice, and only distinct, reproducible, polymorphic and well-resolved bands across all runs were considered

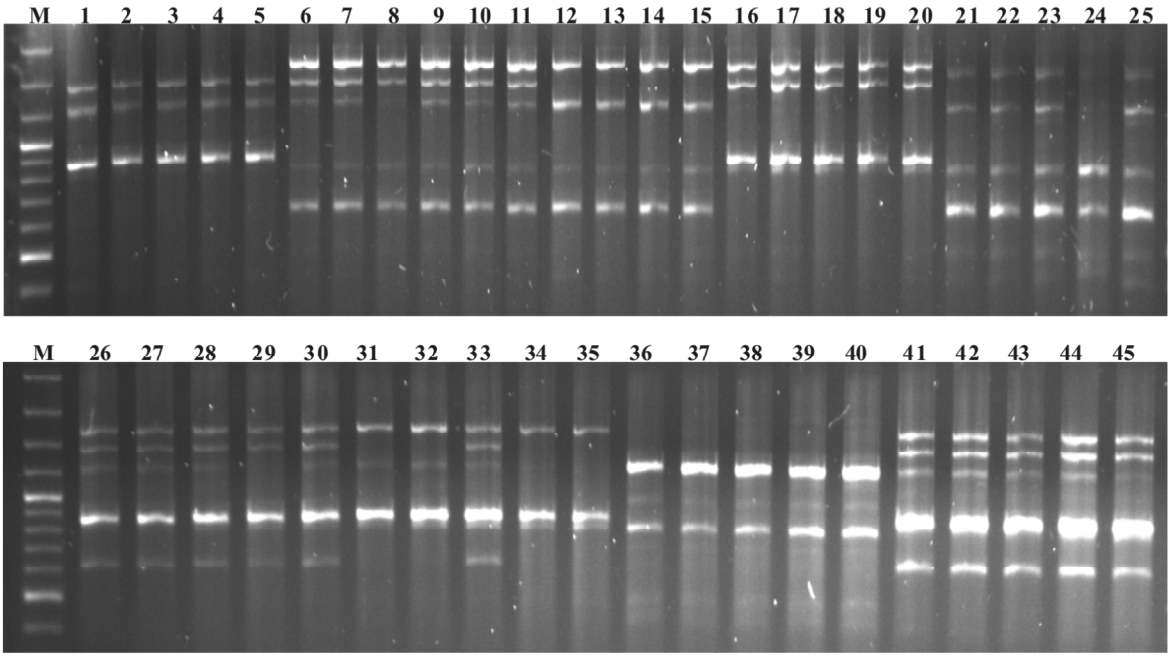

Figure 1: RAPD profiles of *in vitro* propagated potato cultivars (Nos. 1 to 45) generated by the primer OPB09*M* = 100 bp ladder (Fermentas)

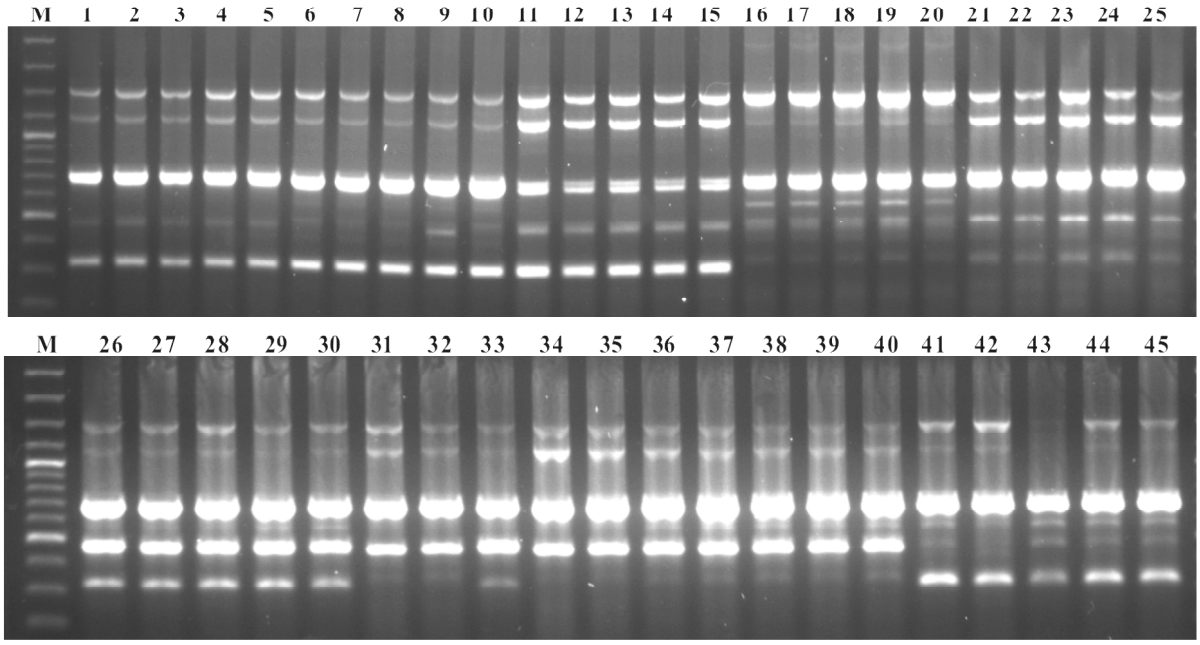

Figure 2: ISSR profiles of *in vitro* propagated potato cultivars (Nos. 1 to 45) generated by the primer ISSRP92. M = 100 bp ladder (Fermentas)

for the analysis. A data matrix was constructed on the basis of presence (1) or absence (0) of bands of the amplified DNA fragments. Missing data were scored as "9". DNA fragments of low visual intensity, which could not be readily distinguished as present or absent, were considered to be ambiguous markers and were not scored. Genetic diversity analysis was performed with the program NTSYS-PC 2.21 (Rohlf, 24). A similarity matrix was calculated by Jaccard's coefficient and the dendrogram was generated using unweighted pair-group method (UPGMA) clustering method. To assess the genetic association of the potato genotypes, a principal component analysis (PCA)

Table 3
Morphological assessment of *in vitro* propagated potato varieties for genetic stability

Potato genotype	*Characters*							
	Microplant height (cm)	*No. of leaves*	*No. of nodes*	*Inter-nodal length (cm)*	*No. of roots*	*Root length (cm)*	*Fresh weight (mg)*	*Dry weight (mg)*
Kufri Bahar-Mother plant	6.5	4.4	4.6	1.4	6.4	10.6	294.6	19.4
Kufri Bahar WGR-10th cycle	8.1	5.8	5.9	1.6	5.9	10.2	142.9	7.2
Kufri Bahar GR-10th cycle	7.2	6.0	6.0	1.0	5.7	8.9	161.6	8.9
Kufri Bahar WGR-20th cycle	8.2	5.8	5.8	1.4	6.4	7.2	264.7	19.1
Kufri Bahar GR-20th cycle	9.7	6.1	6.3	1.5	8.5	5.6	335.0	21.6
SEd	0.43	0.27	0.26	0.11	0.59	0.69	21.4	1.8
CD (0.05)	NS	0.56*	0.54*	NS	NS	1.43*	44.12*	3.7*
Kufri Chandramukhi-Mother plant	6.8	5.2	5.1	1.4	7.3	8.4	406.2	32.3
Kufri Chandramukhi WGR-10th cycle	11.0	6.8	6.5	1.5	4.4	4.4	190.7	12.7
Kufri Chandramukhi GR-10th cycle	13.3	8.1	6.9	2.6	5.5	3.9	246.6	13.6
Kufri Chandramukhi WGR-20th cycle	8.9	5.0	5.2	1.7	7.8	4.38	248.0	17.4
Kufri Chandramukhi GR-20th cycle	10.7	5.1	5.4	2.0	9.3	4.8	418.9	29.2
SEd	0.73	0.44	0.54	0.24	0.83	0.38	34.0	2.3
CD (0.05)	1.5*	0.92*	NS	NS	NS	0.79**	NS	4.8**
Kufri Chipsona-3-Mother plant	4.9	6.7	6.6	0.75	6.5	8.7	410.9	31.8
Kufri Chipsona-3 WGR-10th cycle	8.0	6.7	5.8	1.7	7.0	8.7	187.0	11.9
Kufri Chipsona-3 GR-10th cycle	8.5	6.9	6.1	1.2	6.0	9.1	184.5	11.7
Kufri Chipsona-3 WGR-20th cycle	5.6	6.5	6.7	0.9	7.0	5.6	321.5	23.9
Kufri Chipsona-3 GR-20th cycle	6.3	5.5	5.9	1.1	9.0	3.8	369.7	26.8
SEd	0.45	0.30	0.30	0.13	0.58	0.68	31.9	2.0
CD (0.05)	0.92**	NS	NS	NS	NS	1.4*	65.8*	4.2*
Kufri Girdhari-Mother plant	8.4	3.8	4.3	2.0	4.7	6.5	337.7	24.4
Kufri Girdhari WGR-10th cycle	9.1	6.9	6.7	1.6	5.0	6.0	165.1	9.6
Kufri Girdhari GR-10th cycle	8.0	4.8	4.9	1.6	3.9	7.4	137.1	8.0
Kufri Girdhari WGR-20th cycle	7.7	6.7	6.9	1.2	6.0	4.7	241.3	19.2
Kufri Girdhari GR-20th cycle	8.3	6.5	6.7	1.3	6.9	5.5	269.6	18.4
SEd	0.41	0.35	0.33	0.14	0.54	0.41	22.0	1.7
CD ($p \leq 0.05$)	NS	0.72*	0.68*	0.28*	NS	NS	45.3*	3.53*
Table 3 Continued...								
Kufri Himalini-Mother plant	7.2	4.7	4.7	1.6	6.1	8.7	277.0	24.0
Kufri Himalini WGR-10th cycle	7.9	5.5	5.5	1.5	4.7	7.8	139.5	9.8
Kufri Himalini GR-10th cycle	8.6	5.7	6.1	1.3	5.9	8.7	162.2	9.6
Kufri Himalini WGR-20th cycle	7.2	5.4	5.4	1.3	8.5	4.5	256.7	7.9
Kufri Himalini GR-20th cycle	8.7	5.2	5.7	1.5	8.1	5.4	292.8	20.4
SEd	0.56	0.26	0.27	0.10	0.68	0.36	20.18	1.84
CD ($p \leq 0.05$)	NS	NS	NS	NS	NS	0.74*	41.66*	3.80*

Cont. table 3

Kufri Lauvkar-Mother plant	4.7	5.0	5.0	1.0	7.8	6.7	349.2	25.8
Kufri Lauvkar WGR-10th cycle	8.3	6.0	6.1	1.1	7.0	3.8	163.2	9.9
Kufri Lauvkar GR-10th cycle	7.5	6.1	6.0	1.4	6.4	6.3	171.8	8.2
Kufri Lauvkar WGR-20th cycle	6.5	5.5	5.9	1.1	7.1	5.8	227.4	14.1
Kufri Lauvkar GR-20th cycle	6.8	6.3	6.3	1.1	8.7	5.3	323.6	18.6
SEd	0.47	0.44	0.40	0.09	0.83	0.52	33.0	2.1
CD ($p \leq 0.05$)	0.97*	NS	NS	NS	NS	NS	NS	4.4*
Kufri Pukhraj-Mother plant	5.1	5.6	5.6	0.95	9.3	7.9	434.0	30.6
Kufri Pukhraj WGR-10th cycle	7.4	5.2	4.7	1.6	5.5	7.6	108.3	5.9
Kufri Pukhraj GR-10th cycle	7.2	5.5	5.1	1.5	6.4	5.3	125.8	6.0
Kufri Pukhraj WGR-20th cycle	5.5	4.4	4.5	1.2	6.5	6.4	224.9	12.3
Kufri Pukhraj GR-20th cycle	5.5	5.3	5.3	1.0	5.7	6.7	169.5	9.7
SEd	0.32	0.53	0.44	0.08	0.66	0.54	21.7	1.25
CD ($p \leq 0.05$)	0.67*	NS	NS	0.18*	NS	NS	44.8**	2.58**
Kufri Sindhuri-Mother plant	6.4	4.3	4.2	1.6	7.6	8.8	237.2	18.7
Kufri Sindhuri WGR-10th cycle	12.0	7.2	7.3	1.8	9.2	6.9	211.1	13.9
Kufri Sindhuri GR-10th cycle	11.5	6.6	6.6	2.0	8.8	7.1	228.2	14.6
Kufri Sindhuri WGR-20th cycle	8.9	6.8	6.7	1.3	11.4	6.3	314.2	24.4
Kufri Sindhuri GR-20th cycle	9.2	6.7	7.0	1.3	12.2	5.3	371.6	25.6
SEd	0.33	0.27	0.26	0.12	0.98	0.68	24.5	1.9
CD ($p \leq 0.05$)	0.68**	0.55**	0.54**	NS	NS	NS	NS	NS
Kufri Surya-Mother plant	4.8	5.2	5.1	1.0	9.0	8.4	327.4	22.7
Kufri Surya WGR-10th cycle	7.3	5.2	5.2	1.2	5.0	7.5	114.8	6.3
Kufri Surya GR-10th cycle	8.6	5.1	5.1	2.2	5.0	6.3	98.9	6.0
Kufri Surya WGR-20th cycle	4.3	4.1	4.3	1.0	7.1	7.2	271.6	19.0
Kufri Surya GR-20th cycle	4.5	4.5	4.5	1.0	7.9	7.4	277.8	21.3
SEd	0.63	0.35	0.32	0.21	0.68	0.37	13.9	1.18
CD ($p \leq 0.05$)	0.88*	NS	NS	NS	1.41*	NS	28.8**	2.43**

WGR: Without growth regulators; GR: with growth regulators; SEd: Standard Error of Difference; CD: Critical Difference

was conducted using NTSYS-PC 2.21. PCA plots of the first three resulting principal components were made to assess the accession associations and to identify genetically distinct genotypes.

2.4 Morphological Assessment

After twenty-one days of *in vitro* sub-culturing, observations were recorded on microplant height (cm); number of leaves, nodes and roots; inter-nodal and root length (cm) as well as fresh and dry weight (mg) of each microplant of a test tube. In case of number of roots, only primary roots were counted, as there was secondary branching too. Root length was recorded for the longest root of each plant. Fresh and dry weight was taken for all the three plantlets along with root. For dry weight, microplants from each test tube were dried at 80°C for 48 h in the hot air oven and dry weight was recorded at room temperature.

2.5 Statistical Analysis of Morphological Characters

The experiment was conducted in a factorial completely randomized design. Each treatment comprised four replicates, each replicate consist of four test tubes having three plantlets. As the experiment was repeated once, data were pooled over individual experiments and the average of two times data was analyzed statistically by applying the technique of analysis of variance (ANOVA) as

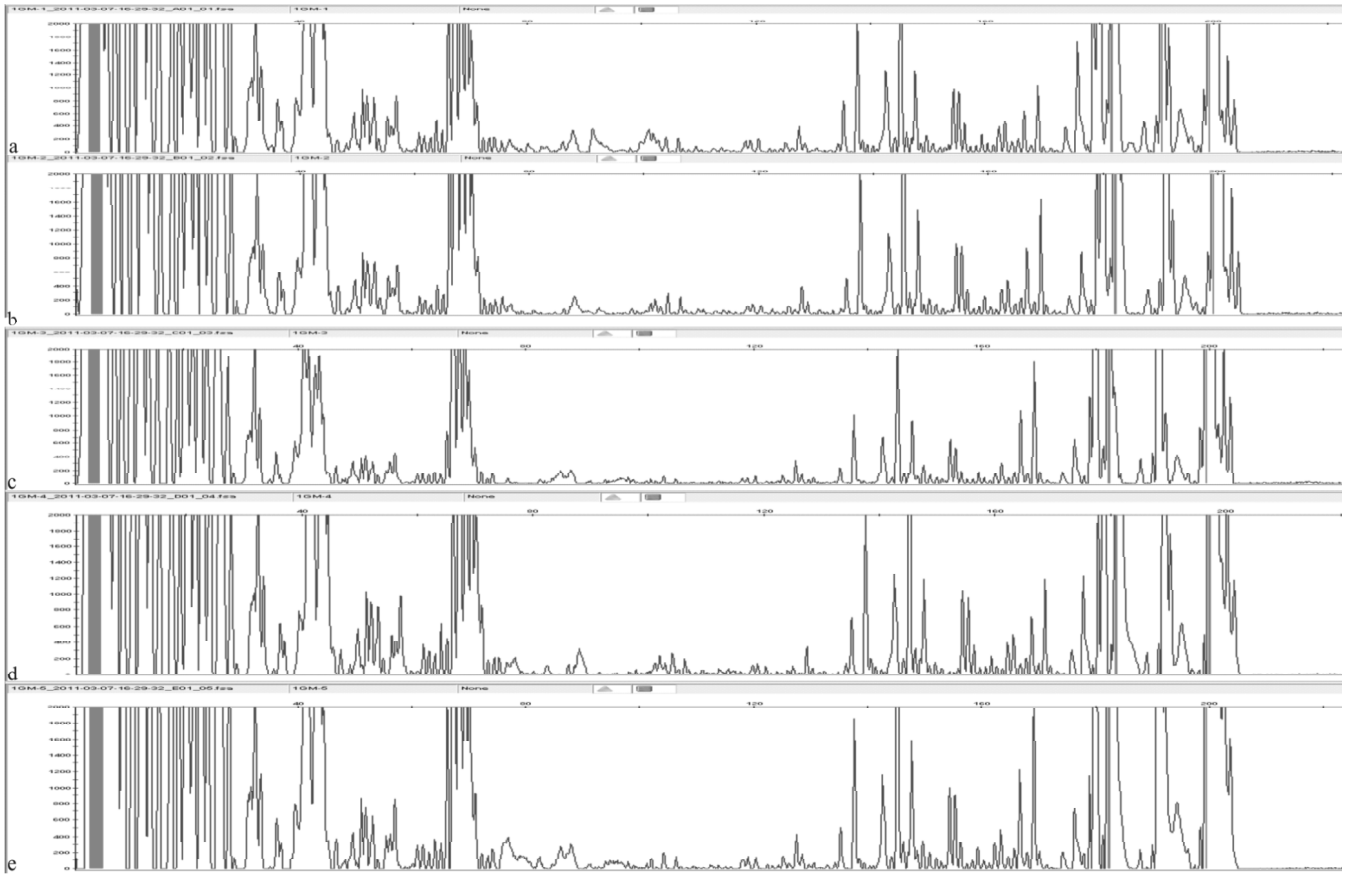

Figure 3 SSR profiles generated by marker STIIKA of *in vitro* propagated potato cultivars (a) KBR1, (b) KBR2, (c) KBR3, (d) KBR4 and (e) KBR5

described by Gomez and Gomez (7). Mean values were calculated and separated using Ftest at 5% level of significance. Statistical analysis was performed the software Windostat (http://www.windostat.org).

RESULTS

To validate genetic fidelity of 10th and 20th cycles regenerants for the seed production, it was essential to ascertain the genetic status of resultant progeny. RAPD, ISSR and SSR markers were employed to characterize the genetic similarity of the mother plant and its regenerants. Out of the 10 RAPD primers, all resulted in two to nine scorable bands per primer. These 10 RAPD primers generated 53 amplicons in total, ranging from 225 to 2000 bp in size. The number of bands in the selected primers varied from 2 (OPB01) to 9 (OPB07), with an average of 5.3 bands per RAPD primer (Table 2). Genetic stability was determined based on the bands appearance as shown in Figure 1 using primer OPB09. Cluster analysis based on the Jaccard's similarity coefficient revealed (Figure 4) 100 % genetic similarity among the mother plants and its derivatives and grouped into nine clusters (I to IX). RAPD cluster analysis revealed true-to-type plants (regenerants) to their corresponding mother plant within the each cluster. In principal component analysis (PCA), variation among the first three principal components accounted for 36.63, 19.14 and 12.05% variation, respectively of the total variation summing up to 67.83%. Nine clusters (I to IX) established by the cluster analysis are clearly separated by the PCA (Figure 5).

Out of nine ISSR markers, all primers resulted in four to ten scorable bands and generated 56 amplicons, ranging from 200 to 3000 bp in size. The number of bands for each primer varied from 4 (ISSR5) to 10 (ISSR2103), with an average of 6.2 bands per ISSR primer (Table 2). Genetic stability was determined based on the bands appearance and they showed identical to mother plant as shown in Figure 2 using primer ISSRP92. Cluster analysis

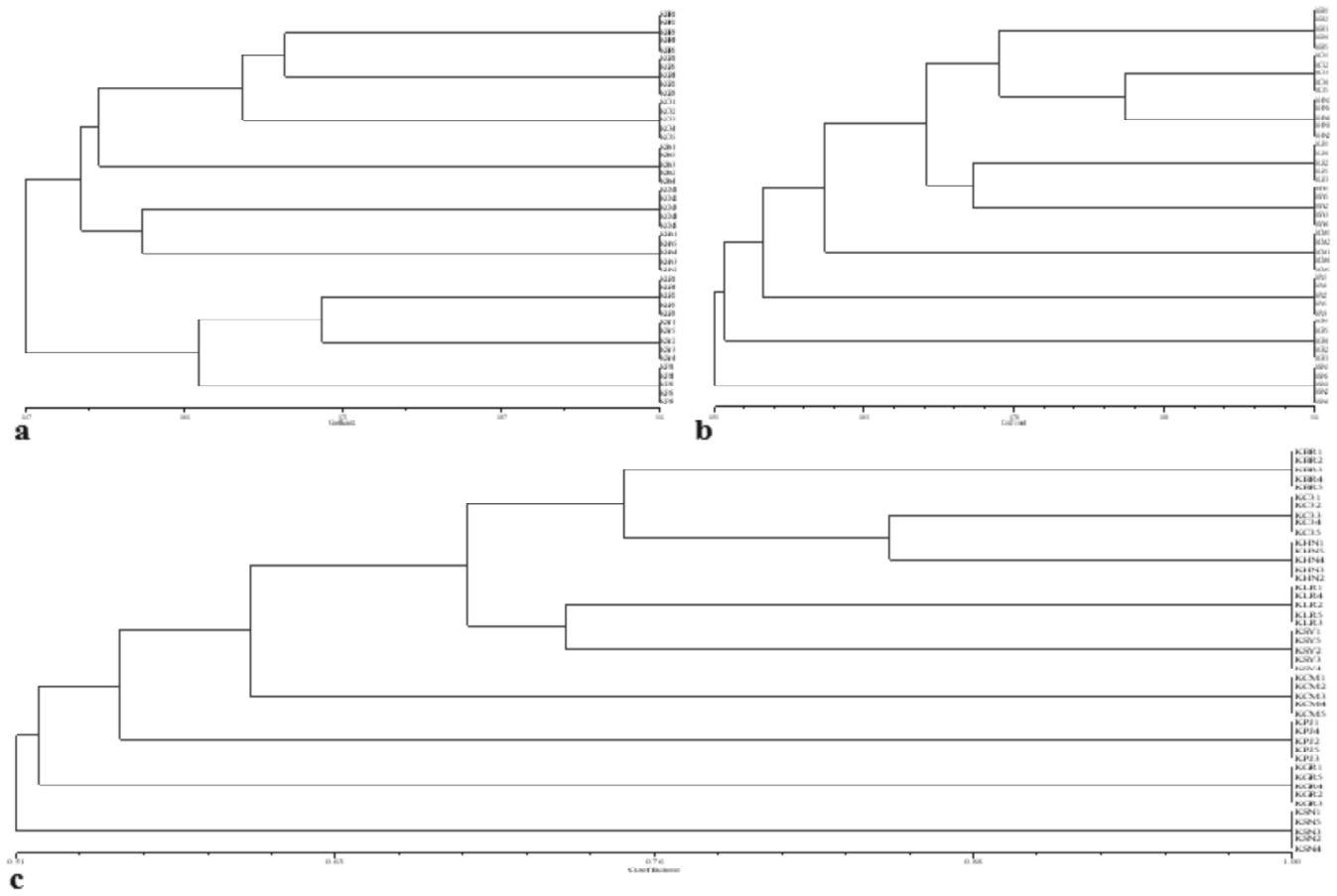

Figure 4 Cluster analysis of *in vitro* propagated potato cultivars based on the Jaccard's coefficient by (a) RAPD, (b) ISSR, and (c) SSR markers

based on the Jaccard's similarity coefficient revealed (Figure 4) 100 % genetic similarity among the mother plants and its derivatives and grouped into nine clusters (I to IX). ISSR cluster analysis revealed true-to-type plants (regenerants) to their corresponding mother plant within the each cluster. In principal component analysis (PCA), variation among the first three principal components accounted for 40.66, 24.73 and 11.28% variation, respectively of the total variation summing up to 76.68%. Nine clusters (I to IX) established by the cluster analysis are clearly separated by the PCA (Figure 5).

Out of five SSR primers, all resulted in six to ten scorable bands per primer. These 5 SSR primers generated 43 amplicons in total, ranging from 118 to 304 bp in size. The number of bands in the selected primers varied from 6 (STM1053) to 10 (STM5127 and STM5114), with an average of 8.6 bands per SSR primer (Table 2). To illustrate, Figure 3 shows SSR profiles generated by marker STIKA of mother plant KBR1 (1) identical to KBR2 (2), KBR3 (3), KBR4 (4) and KBR5 (5). Cluster analysis based on the Jaccard's similarity coefficient revealed (Figure 4) 100% genetic similarity among the mother plants and its derivatives and grouped into nine clusters (I to IX). SSR cluster analysis revealed true-to-type plants (regenerants) to their corresponding mother plant within the each cluster. In principal component analysis (PCA), variation among the first three principal components accounted for 30.00, 16.22 and 12.28% variation, respectively of the total variation summing up to 58.51%. Nine clusters (I to IX) established by the cluster analysis are clearly separated by the PCA (Figure 5).

MORPHOLOGICAL ASSESSMENT

Results of the morphological assessment of *in vitro* propagated potato genotypes are outlined in Table 3. In the cultivar Kufri Bahar sub-culturing cycles significantly influenced number of leaves and nodes, root length, fresh and dry weight of the

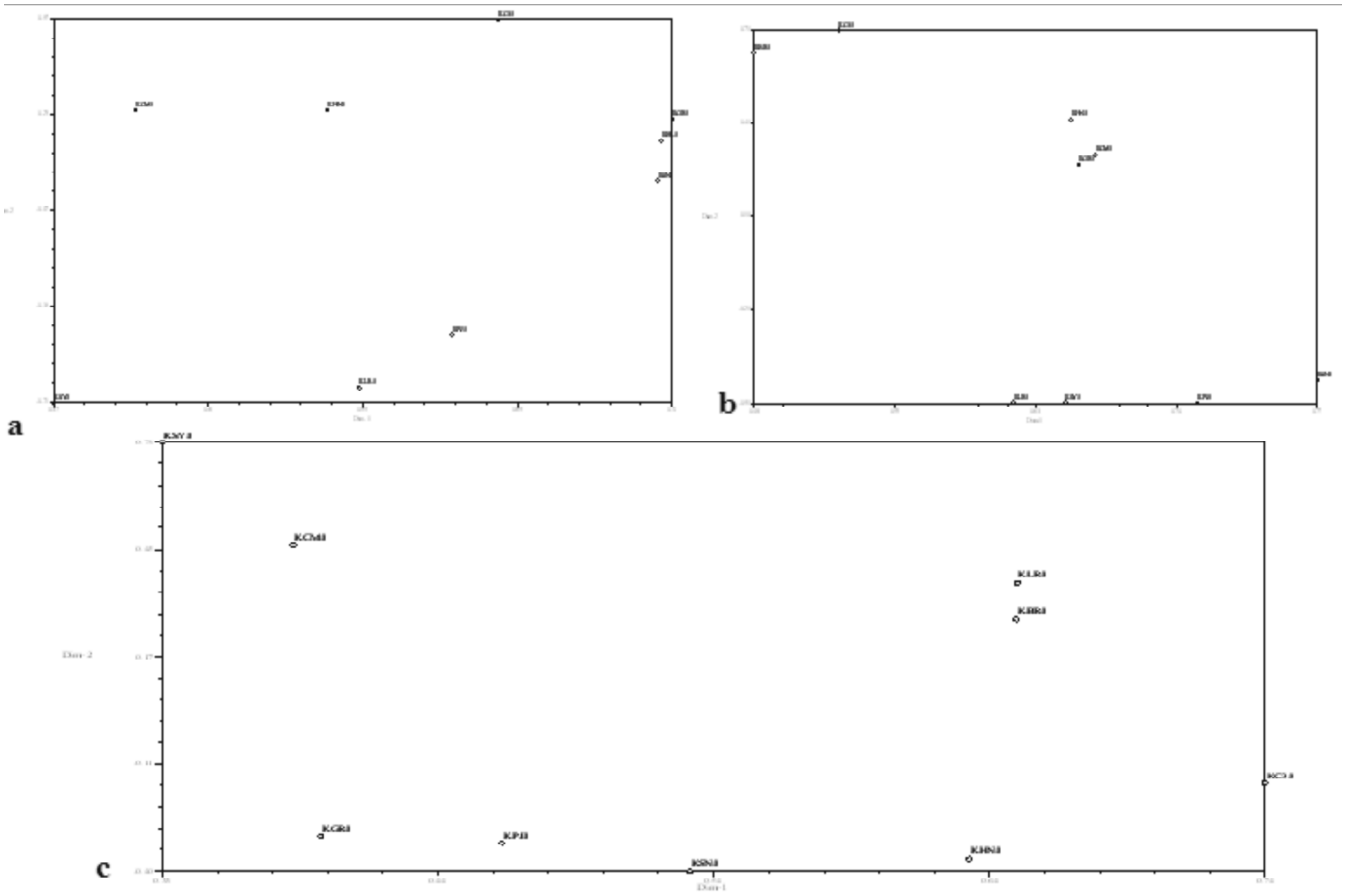

Figure 5 Principal Component Analysis (PCA) of *in vitro* propagated potato cultivars based on the Jaccard's coefficient by (a) RAPD, (b) ISSR, and (c) SSR markers

microplants. Cultures grown in 10th and 20th cycle produced at par but significantly more no. of nodes as compared to 1st cycle. However, increased sub-culturing cycle significantly reduced the no. of roots. First and 20th sub-culturing cycles resulted accumulation of maximum fresh and dry weight whereas the 10th cycle the minimum. In the cultivar Kufri Chandramukhi sub-culturing cycles had significant effect on microplant height, number of leaves, root length and dry weight. Among the sub-culturing cycles, cultures grown in 10th cycle produced significantly maximum microplant height and no. of leaves whereas, 1st cycle did so for no. of roots and dry weight.

In the cultivar Kufri Chipson-3 sub-culturing cycles significantly affected microplant height, root length, fresh and dry weight. Cultures grown in 10th cycle produced significantly maximum microplant height and which was minimum in 1st cycle. However, increased sub-culturing cycle significantly reduced the no. of roots. But there was no trend in fresh and dry weight due to sub-culturing cycles. First sub-culturing cycles resulted accumulation of maximum fresh and dry weight as compared to 20th cycle. In the cultivar Kufri Girdhari, sub-culturing cycles had significant effect on number of leaves and nodes, inter-nodal length, fresh and dry weight. Increased sub-culturing cycle significantly enhanced the number of leaves as well as nodes but, the same had negative effect on inter-nodal length, fresh and dry weight.

In the cultivar Kufri Himalini sub-culturing cycles significantly influenced root length fresh and dry weight of microplants. First sub-culturing cycle produced significantly maximum root length, fresh and dry weight as compared to other cycles studied. In the cultivar Kufri Lauvkar, sub-culturing cycles had significant effect only on microplant height and dry weight. Cultures grown in 10th cycle produced significantly maximum microplant height and which was minimum in 1st cycle. First sub-culturing

cycles resulted accumulation of maximum dry weight and 10th cycle the minimum. In the cultivar Kufri Pukhraj sub-culturing cycles had significant effect on microplant height, inter-nodal length and fresh as well as dry weight. Cultures grown in 10th cycle produced significantly maximum microplant height as well as inter-nodal length whereas, 1st cycle the minimum. First sub-culturing cycle resulted accumulation of maximum fresh and dry weight as compared to 10th cycle.

In the cultivar Kufri Sindhuri sub-culturing cycles significantly influenced the microplant height, number of leaves and nodes. Cultures grown in 10th cycle produced significantly maximum microplant height, no. of leaves and nodes whereas, 1st cycle the minimum. In the cultivar Kufri Surya sub-culturing cycles had significant effect on microplant height, number of roots and fresh as well as dry weight. Among the sub-culturing cycles, cultures grown in 10th cycle produced significantly maximum microplant height whereas, 1st cycle the minimum. Microplants grown in first sub-culturing cycle recorded maximum no. of roots, fresh and dry weight as compared to other two cycles studied.

DISCUSSION

Considering the importance of ensuring genetic stability of micro-propagated plants in any tissue-culture-based seed production programme, it is imperative to choose a procedure that does not induce variation. Occurrence of genetic variations is a serious problem in micropropagation of crop species because of their unpredictable nature. The variations generated during tissue culture processes are generally caused by chromosomal rearrangements (Rahman and Rajora, 23). Detection and the analysis of genetic variation can help in understanding the molecular basis of various biological phenomenons in plants. Variations induced in tissue cultured plants are most likely to be reflected in the banding profiles developed by different marker systems (Phillips *et al.*, 22). To uncover genetic changes, in this study we used RAPD, ISSR and SSR profiles with mother plant and its derivatives.

Molecular markers analyses demonstrated that the mother plant and its clonal derivatives could be grouped together in a single cluster with 100% similarity and indicated a "true-to-type" progeny. PCA analysis also demonstrated that the mother plant and its derivatives of the corresponding clusters were plotted together. The present data are similar to reports of others. There are a number of findings in the literature which report similar results for detection of genetic fidelity using molecular markers in many crops such as *Solanum aculeatissimum* (Ghimire *et al.*, 5), *Olea europaea* (Leva *et al.*, 13), *Saccharum officinarum* (Tawar *et al.*, 28), *Dendrocalamus hamiltonii* (Agnihotri *et al.*, 1) and *Capparis deciduas* (Tyagi *et al.*, 30). For example, Kumar *et al.* (12) assessed genetic fidelity with similarity level of 100% of micro-propagated plants and mother plants of jojoba using RAPD and ISSR markers and indicated axillary bud multiplication can also be used as one of the safest modes for the production of true-to-type plants. Bhatia *et al.* (3) evaluated genetic fidelity with 100% similarity of in vitro propagated plants and mother plant of gerbera using RAPD and ISSR marker. Zarghami *et al.* (33) evaluated genetic stability upto a level of 97-100% similarity in cryopreserved and non-cryopreserved potato using AFLP markers. Dann and Wilson (4) observed genetic (8.75-15.63% polymorphisms) and epigenetic (12.56-26.13% polymorphisms) variations among regenerants of potato derived from long-term nodal tissue culture and cell selection. Aversano *et al.* (2) demonstrated that under in vitro culture conditions *Solanum* genotype affects the integrity of the genome and absence of polymorphism at plastid level confirms the greater genetic stability of cytoplasmic DNA.

Extensive research has been conducted on genetic and epigenetic changes that may be attributed to the somaclonal variations during tissue culture. It was observed that presence or absence of variations during tissue culture depends upon the source of explant and the mode of regeneration, including levels of growth substances that are used (Martin *et al.*, 16). Plants regenerated from axillary buds or from other meristematic tissue showed the lowest tendency for genetic variation (Joshi and Dhawan, 9).

In the clonal regeneration, sub-culturing cycles influenced morphological characters in the various cultivars differently. Genetically controlled *in vitro* response has already been reported in potato previously (Gopal *et al.*, 8; Sharma *et al.*, 25;

Venkatasalam *et al*., 32). In general microplant height, inter-nodal length, no. of leaves and nodes increased in 10th cycle as compared to first sub-culturing cycle and further reduced during 20th cycle but 1st and 20th cycle the same was found to be statistically at par with each other. Increased sub-culturing cycles had negative effect on morphological characters like no. of roots, root length and fresh as well as dry however there was no. significant difference between 10th and 20th cycle. Therefore, true-to-type nature of the *in vitro* propagated plants can be multiplied up to 20 cycles without any effect on morphological characters. Our results revealed that micropropagation of potato using leafy nodal cuttings maintains the genetic integrity even up to 20 cycles under certain in vitro conditions and is a safe method for tissue-culture-based seed potato production.

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