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Analysis of genetic stability of Micropropagated potato Microtubers using random amplified polymorphic DNA (RAPD) markers

Shubhendra Singh Chauhan, Pankaj Kumar Singh and Pragati Mishra

Abstract
Micropropagation is a promising technique for development of pathogen and virus free microtubers which can be used as better "potato seed" than those produced conventionally. Genetic stability and biosafety of such seeds is matter of concern before its consumption. Genetic fidelity of micropropagated microtubers of potato (*Solanum tuberosum*), developed as *in vitro* cultured pathogen free "potato seeds", was evaluated using Random amplified polymorphic DNA (RAPD) markers. Nine arbitrary decamers were used to amplify DNA from *in vivo* control tuber and *in vitro* micropropagated microtubers to assess the genetic fidelity. RAPD profiles from all the micropropagated microtubers were monomorphic and similar to those of field grown control tubers. There was no detectable variation within the micropropagated microtubers which suggests its genetic stability.

Keywords: micropropagation, genetic fidelity, RAPD, microtubers, genetic markers

Introduction
Micropropagation is a known technique for the production of pathogen free plants (Loebenstein *et al*. 2001; Faccioli and Zoffoli, 1998) [20, 8] from small healthy explants using tissue culture. It is very useful for agriculture based economy. Though the micropropagated plants are theoretically identical to parent plant but, there is a possibility of genetic and/or epigenetic modifications leading to genetic variation, widely known as somaclonal variation (Larkin and Scowcroft, 1981) [16]. Activation of transposable elements (McClintock, 1984; Hirochika *et al*. 1996) [22, 13], DNA hypomethylation (Jaligot *et al*. 2000) [14], genome adaptation to different regulatory microenvironments (Bogani *et al*. 1996) [4], and the presence of DNA instability hot spots (Linacero *et al*. 2000) [18] are some of the causes of somaclonal variation. Genetic alterations assosciated with phenotypic alterations were reported in micropropagated plants of several species from *Pelargonium, Ananas, Phalaenopsis, Arachis, Musa,* and *Saintpau- lia genera* (Cassels *et al*. 1997; Das and Bhowmik, 1997; Chen *et al*. 1998; Eapen *et al*. 1998; Grajal-Martfn *et al*. 1998; Paek and Hahn, 1999) [5, 7, 6, 31, 12, 25]. Early stage identification of variants is essential to avoid propagation of mutant plants (Rival *et al*. 1998; Jaligot *et al*. 2000) [29, 14]. Use of random amplified polymorphic DNA (RAPD) marker is a pertinent approach in this regard.
Potato (*Solanum tuberosum* L.) is the fourth largest crop in world with 381.68 million tonnes annual production (FAO, 2014). It produces the largest quantity of carbohydrates per day per unit area among the food crops (Zaag and Horton, 1983) [38] and is marked as a chief energy food crop. India is the second largest producer of potato after China. Conventional "in fields" production of potato seed tubers is costly. The produce is lower in quality and often infected with various bacterial, fungal and viral pathogens (Priou and Mahjoub, 1999; Khan, 1981) [28]. These diseases and their chemical remedies are hazardous to economy and environment respectively.
Micropropagation produces virus free potato seed tubers (Naik and Karihaloo, 2007) [23]. Further, thermotherapy and chemotherapy techniques used during micropropagation can eliminate bacterial and fungal pathogens (Faccioli and Zoffoli, 1998) [8]. This technique has helped to increase the world potato yield (Naik and Karihalorolo, 2007) [23] from 17.4 tonnes/ha in 2004 to 19.98 tonnes/ha in 2014 (FAO, 2014). But, such tubers should pass the strict tests for genetic stability and biosafety before their consumption.
Randomly amplified polymorphic DNA (RAPD) is a simple and effective assay technique for screening of genomic alterations among tissue culture derived plants (Gallego *et al*. 1997; Shoyama *et al*. 1997) [9, 33]. It is proven to be quite efficient in evaluating natural genetic diversity in several plant species and is used extensively (Martin *et al*. 2004; Aversano *et al*. 2009; Bhattacharya *et al*. 2010; Liu *et al*. 2011; Pathak and Dhawan, 2012) [21, 2, 3, 19, 26].

Present study aims to determine the genetic stability of micropropagated potato seed microtubers developed in northern India. This study will help in the validation of efficiency and safety of micropropagation technique for use in present cultivar and will be an iron gate for the commercial utilization of produced microtubers.

Methods and Materials

Plant material: Micropropagated seed tubers for potato cv. chipsona were developed and tested as a primary source for commercial production. Procedure was standardized for the cultivar following series of subcultures from healthy tuber derived explant. One genotype of cv. chipsona was selected as mother explant and obtained from the farm yard, Department of Horticulture, Allahabad School of Agriculture, Sam Higginbottom University of Agriculture, Technology and Sciences (SHUATS), Allahabad, India. Ten micropropagated seed tubers were randomly selected for genetic stability test as described in culture conditions.

Culture conditions: Plantlets were multiplied using leafy nodes of the tubers in the propagation medium (MS medium + 30 gl−1 sucrose + 7 gl-1 agar) at 20 °C in an environment controlled chamber. Twenty-days old "*in vitro*" plants were induced for microtuber production on the tuber inducing medium (MS medium + 80 gm/1 sucrose + 10 ml/l BAP) without agar. Out of these developed microtubers, ten were randomly selected and named T1, T2, T3, T4, T5, T6, T7, T8, T9 and T10. The leave of plantlets regenerated from these tubers was used for genetic stability analysis. Tissue culture experiments were done at Plant Tissue Culture Laboratory, Department of Molecular and Cellular Engineering, SHUATS, Allahabad.

DNA extraction: Total genomic DNA was extracted from young, unexpanded leaves using a cetyltrimethylammonium bromide (CTAB) procedure (Bousquet *et al*. 1990). Approx. 200 mg 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. The extraction buffer consisted of 2% (w/v) CTAB (cetyltrimethyl amonium bromide, Sigma), 1.4 M NaCl, 20 mM EDTA, 100 mM Tris-HCl pH 9.5, and 0.2% (v/v) β-mercaptoethanol. Incubation of the obtained homogenate at 60 ºC for 2 h was followed by extraction using equal volume of chloroform/isoamyl alcohol (24:1 v/v) and centrifugation at 10,000 x *g* for 20 min. Equal volume of isopropanol was added for DNA precipitation. Precipitate was centrifuged at 10,000 x *g* for 10 min and the DNA pellet obtained 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. After gel electrophoretic analysis on 1% agarose gel and spectrophotometric quanification for purity and integrity, the isolated DNA samples were finally diluted with sterile TE buffer to get the final concentration of 25 ng μl^{-1}. The diluted DNA was subsequently used for polymerase chain reaction (PCR) amplification.

PCR amplification: Nine random decamer RAPD primers (Sigma-Aldrich Chemicals, St. Louis, USA) were used for polymerase chain reaction (PCR), following the protocol of Williams *et al*. (1990), with minor modifications. Each PCR reactions mixture (25 µl) contained 2.5 µl of 10× PCR buffer (Thermo Fisher, USA), 2.0 µl of 1.25 mM dNTPs each (Thermo Fisher, USA), 0.5µM of primer, 0.5 units of Taq DNA polymerase (Thermo Fisher, USA), 2.5 mM $MgCl_2$, and 25ng (1 µl) of genomic DNA. PCR amplification was performed in MJ Mini PCR cycler (BIO-RAD, USA) programmed for initial denaturation at 95 °C for 5 minutes followed by 35 cycles of 1 min denaturation at 95 ºC, 1 min annealing at 37 ºC and 2 min of extension at 72 ºC. Cycles were followed by a final extension at 72 ºC for 10 min. The amplified products were subjected to gel electrophoresis using gel prepared with 1.2% (w/v) agarose (Sigma, USA) in 0.5X TBE buffer and stained with ethidium bromide. DNA ladder plus 100bp "GeneRuler" (Fermentas life science, Germany) was used as molecular size marker. Amplified products run on gel were observed in Alphaimager Gel Documentation System (Alpha Innotech Corporation, USA). All the reactions were repeated twice for confirmation.

Scoring and Data Analysis: Amplified markers were scored on the basis of presence (1) or absence (0) of bands. Bands of low visual intensity, unable to distinguish were not scored. Similarity matrix, using all scorable bands, was calculated by Jaccard's coefficient and the dendrogram was generated using unweighted pair-group (UPGMA) clustering method (Sneath and Sokal, 1973). Genetic diversity analysis was performed with the program NTSYS-PC 2.02i (Numerical Taxonomy System for Personal Computers, version 2.02i) (Rohlf, 1993) [30]. The polymorphism information content (PIC) was calculated as:

$$PIC_i = 1 - {}^{n}\sum_{j=1} P^2_{ij}$$

P_{ij} is the frequency of *j*th allele for marker i and the summation extends over n alleles. The calculation was based on the number of bands in RAPD.

Results

Micropropagated microtubers developed from potato (*Solanum tuberosum* L.) cv. Chipsona was analysed for genetic stability compared to mother plant, using RAPD assay. Nine primers were randomly selected (OPA 02-05 and OPB 05-10) for the assay. All of these primers generated 3 to 8 bands per primer. A total of 55 amplicons were generated per sample ranging from 200bp to 2800bp in size. The number of bands in different selected primers varied from 5 (OPA-04 and OPB-10) to 8 (OPB-08) with an average of 6.1 bands per primer (Table 1). Genetic stability determined on the basis of band presence and absence revealed genetic identity of the micropropagated microtubers to the mother plant. Band patterns obtained by RAPD assay by primer OPA-03 and OPB-08 on agarose gel (Fig 1) were identical in the control (mother explants "C") and sample tubers (T1-T10). Calculation of the Jaccard's similarity coefficient revealed 100% genetic similarity among the mother plants and derived micotubers of chipsona cultivar and thus dendrogam couldn't be generated.

Table 1: RAPD amlicon profiles of "*in vitro*" micropropagated microtubers of potatao

S. No.	Primers	5'-3' Sequence	Band size range	Total No. of Bands	Size of each band
1	OPA-02	TGCCGAGCTG	400-2000	6	400, 700, 900, 1200, 1500, 2000
2	OPA-03	AGTCAGCCAC	450-1800	6	450, 700, 850, 1000, 1500,1800
3	OPA-04	AATCGGGCTG	500-1900	5	500, 700, 850, 1000, 1900
4	OPA-05	AGGGGTCTTG	400-2000	6	400, 700, 900, 1200, 1500, 2000
5	OPB-06	TGCTCTGCCC	200-700	6	300, 400, 450, 500, 660

6	OPB-07	GGTGACGCAG	150-1000	7	220, 430, 520, 610, 725, 900, 1000
7	OPB-08	GTCCACACGG	350-1600	8	350, 550, 625, 675, 900, 1000, 1600
8	OPB-09	TGGGGGACTC	380-3000	6	380, 620, 960, 1480, 2000, 2800
9	OPB-10	CTGCTGGGAC	325-700	5	300, 350, 500, 550, 680

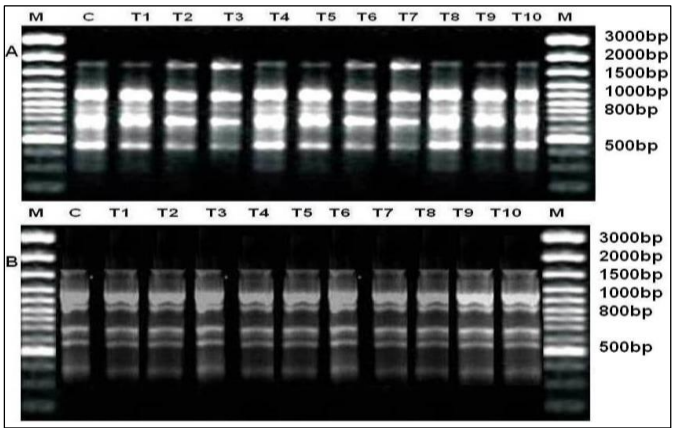

Fig 1: RAPD profiles of micropropagated microtubers of potato using (A) Primer OPA-03 (B) OPB-08. "C" represents mother plant whereas T1-10 represents ten samples of micropropagated tubers. Lane "M" represents ladder Gene Ruler 100bp plus (Fermentas life science, Germany)

Discussion

Micropropagation has been proved to be an efficient technique for the development of pathogen free plants which are identical to the parent plant. This technique is widely utilized in many plant species and thus has helped fight many plant diseases. It has been proved a boon for the growing economies like India by helping the micropropagation of potato seed tubers. But, there are chances of genetic alteration due to some genetic and epigenetic effects, action of transposons, chemical or environment induced mutations, DNA hypomethylation (McClintock, 1984; Hirochika *et al*. 1996, Jaligot *et al*. 2000, Bogani *et al*. 1996) [22, 13, 14, 4]. Genetic variation was observed in many plant species (Cassells *et al*. 1997; Das and Bhowmik, 1997; Chen *et al*. 1998; Eapen *et al*. 1998; Grajal-Martfn *et al*. 1998; Paek and Hahn, 1999) [5, 7, 6, 31, 12, 25]. Thus, genetic stability analysis of micropropagated plants is an essential step before approval of consumption. Moreover, detection and analysis of genetic variation can help in understanding the molecular basis of various biological phenomenon 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*. 1994) [27].

RAPD analysis was used in the present study to detect the genetic changes between mother plant and microtubers of potato developed by micropropagation. The analysis found 100% genetic similarity between the tested plants. The present findings are in concordance with other reports in various plant species. Similarity in genetic fidelity was observed in various cultivars of Indian potato (*Solanum tuberosum*) (Tiwari *et al*. 2013) [36] Solanum species (Aversano *et al*. 2009) [2], *Solanum aculeatissimum* (Ghimire *et al*. 2012) [10], Olea europaea (Leva and Petruccelli, 2012) [17], *Saccharum officinarum* (Tawar *et al*. 2008) [35], *Dendrocalamus hamiltonii* (Agnihotri *et al*. 2009) [1], *Capparis deciduas* (Tyagi *et al*. 2010) [37], Vanilla species (Sreedhar *et al*. 2007) [37], *Dioscorea bulbifera* (Narula *et al*. 2007) [24] and Musa species (Lakshmanan *et al*. 2007) [15]. 97-100% genetic similarity to mother plant was observed in "*in vitro*" developed potatoes by (Zarghami *et al*. 2008) [39]. Selection of leafy buds, controlled media and "*in vitro*" conservation can be the reason for genetic similarity of obtained microtubers in present study. However, tests using other robust markers like ISSR, AFLP can be done for confirmation.

References

1. Agnihotri RK, Mishra J, Nandi SK. Improved *in vitro* shoot multiplication and rooting of Dendrocalamus hamiltonii Nees Arn. Ex Munro: production of genetically uniform plants and field evaluation. Acta Physiology Plant. 2009; 31:961-967.
2. Aversano R, Savarese S, Nova JMD, Frusciante L, Punzo M, Carputo D. Genetic stability at nuclear and plastid DNA level in regenerated plants of Solanum species and hybrids. Euphytica. 2009; 165:353-361.
3. Bhattacharya S, Bandopadhyay TK, Ghosh PD. High frequency clonal propagation of *Cymbopogon martinii* var motia (Palmarosa) through rhizome culture and true to type assessment using ISSR marker. Journal of Plant Biochemistry and Biotechnology. 2010; 19:27-274.
4. Bogani P, Simoni A, Lio P, Scalpi A, Buiatti M. Genome flux in tomato cell clones cultured *in vitro* in different physiological equilibria. II. A RAPD analysis of variability. Genome. 1996; 39:846-853.
5. Cassells AC, Croke JT, Doyle BM. Evaluation of image analysis, flow cytometry and RAPD analysis for the assessment of somaclonal variation and induced mutation in tissue cultured derived Pelargonium plants. Journal of Applied Botany. 1997; 71:125-130.
6. Chen WH, Chen TM, Fu YM, Hsieh RM, Chen WS. Studies on somaclonal variation in Phalaenopsis. Plant Cell Report. 1998; 18:7-13.
7. Das RK, Bhowmik G. Some somaclonal variants in pineapple (*Ananas comosus* (L.) Merr.) Plants obtained from different propagation techniques. International Journal of Tropical Agriculture. 1997; 15:95-100.
8. Faccioli G, Zoffoli R. Fast eradication of potato virus X (PVX) and potato virus S (PVS) chemotherapy, Phytopathologia Mediterranea. 1998; 37:9-12.
9. Gallego FJ, Martinez I, Celestino C, Toribio M. Testing somaclonal variation using RAPDs in *Quercus suber* L. somatic embryos. International Journal of Plant Sciences. 1997; 158(5):563-567.
10. Ghimire BK, Yu CY, Chung IM. Direct shoot organogenesis and assessment of genetic stability in regenerants of Solanum aculeatissimum Jacq. Plant Cell Tissue Organ Culture. 2012; 108:455-464.
11. Goto S, Thakur RC, Ishii K. Determination of genetic stability in long-term micropropagated shoots of Pinus thunbergii Parl. Using RAPD markers. Plant Cell Report. 1998; 18:193-197.
12. Grajal-Martin M, Siverio-Grillo G, Marrero-Dominguez A. The use of randomly amplified polymorphic DNA (RAPD) for the study of genetic diversity and somaclonal variation in Musa. Acta Horticulturae. 1998; 490:445-454.
13. Hirochika H, Sugimoto K, Otsuki Y, Tsugawa H, Kanda M. Retrotransposons of rice involved in mutations

induced by tissue culture. Proceedings of the National Academy of Science of the USA. 1996; 93:7783-7788.
14. Jaligot E, Rival A, Beulé T, Dussert S, Verdeil JL. Somaclonal variation in oil palm (Elaeis guineensis Jacq.): the DNA methylation hypothesis. Plant Cell Report. 2000; 19:684-690.
15. Lakshmanan V, Venkataramareddy SR, Neelwarne B. Molecular analysis of genetic stability in long-term micropropagated shoots of banana using RAPD and ISSR markers. Electronic Journal of Biotechnology. 2007; 10:106-113.
16. Larkin PJ, Scowcroft WR. Somaclonal variation- A novel source of variability from cell cultures for plant improvement. Theoretical and Applied Genetics. 1981; 58:197-214.
17. Leva AR, Petruccelli R. Monitoring of cultivar identity in micropropagated olive plants using RAPD and ISSR markers. Biologia Plantarum. 2012; 56:373-376.
18. Linacero R, Freitas-Alves E, Vasquez AM. Hot spots of DNA instability revealed through the study of somaclonal variation in rye. Theoretical and Applied Genetics. 2000; 100:506-511.
19. Liu LS, Li R, Zhao Y, Wen CL, Ren S, Guo YD. High efficiency regeneration and genetic stability analysis of somatic clones of Gynura bicolor DC. African Journal of Biotechnology. 2011; 10:10380-10386.
20. Loebenstein G, Berger PH, Brunt AA, Lawson RH. Virus and Virus-like Diseases of Potatoes and Production of Seed-Potatoes, Kluwer Academic Publishers, 2001.
21. Martin M, Sarmento D, Oliveira MM. Genetic stability of micropropagated almond plantlets, as assessed by RAPD and ISSR markers. Plant Cell Report. 2004; 23:492-496.
22. McClintock B. The significance of responses of the genome to challenge. Science. 1984; 226:792-801.
23. Naik PS, Karihaloo JL. Micropropagation for the production of quality potato seed in Asia-Pacific. Asia Pacific Consortium on agricultural biotechnology, 2007.
24. Narula A, Kumar S, Srivastava PS. Genetic fidelity of *in vitro* regenerants encapsulation of shoot tips and high diosgenin content in *Dioscorea bulbifera* L. a potential alternative source of diosgenin. Biotechnology Letters. 2007; 29:623-629.
25. Paek KY, Hahn EJ. Variations in African violet 'Crimson Frost' micropropagated by homogenized leaf tissue culture. Hort Technology, 1999, 625-628.
26. Pathak H, Dhawan V. ISSR assay for ascertaining genetic fidelity of micropropagated plants of apple rootstock Merton 793. *In Vitro* Cellular & Developmental Biology Plant. 2012; 48:137-143.
27. Phillips RL, Kaeppler SM, Olhoft P. Genetic instability of plant tissue cultures: breakdown of normal controls. Proceedings of the National Academy of Sciences U. S. A. 1994; 91:5222-5226.
28. Priou S, Mahjoub MEl. Bacterial and fungal diseases in the major potato-growing areas of Tunisia. EPPO Bulletin. 1999; 29:167-171.
29. Rival AB, Tregear J, Verdiel JL, Richaud F, Beule T, Duval Y. Molecular search for mRNA and genomic markers of the oil palm 'mantled' somaclonal variation. In: Drew RA. ed. Proc. Int. Symp. Biotechnology Tropical & Subtropical Species. Acta Horticulturae. 1998; 461:165-171.
30. Rohlf FJ (1993). NTSYS-pc. Numerical taxonomy and multivariate analysis system: version 1.8. Applied Biostatistics, New York.
31. Eapen S, Tivarekar S, George L. Thidiazuron induced shoot regeneration in pigeonpea (*Cajanus cajan* L.). Plant Cell Tissue and Organ Culture. 1998; 53:217-220.
32. Sneath PHA, Sokal RR. Numerical taxonomy the principles and practice of numerical classification, 1973.
33. Shoyama Y, Zhu XX, Nakai R, Shiraishi S, Kohda H. Micropropagation of Panax notoginseng by somatic embryogenesis and RAPD analysis of regenerated plantlets. Plant Cell Report. 1997; 16:450-453.
34. Sreedhar RV, Venkatachalam L, Bhagyalakshmi N. Genetic fidelity of long-term micropropagated shoot cultures of vanilla as assessed by molecular markers. Biotechnology Journal, 2007; 2:1007-1013.
35. Tawar PN, Sawant RA, Dalvi SG, Nikam AA, Kawar PG, Devarumath RM. An assessment of somaclonal variation in micropropagated plants of sugarcane by RAPD markers. Sugar Tech. 2008; 10:124-127.
36. Tiwari JK, Singh BP, Gopal J, Poonam Patil VU. Molecular characterization of the Indian Andigena potato core collection using microsatellite markers. African journal Biotechnology. 2013; 12:24-36.
37. Tyagi P, Khanduja S, Kothari SL. *In vitro* culture of Capparis decidua and assessment of clonal fidelity of the regenerated plants. Biologia Plantarum. 2010; 54:126-130.
38. Zaag DE, Harton D. Potato prospective with special reference to the tropics and sub-tropics. Potato Research. 1983; 26:323-328.
39. Zarghami R, Pirseyedi M, Hasrak S, Sardrood BP. Evaluation of genetic stability in cryopreserved Solanum tuberosum. African Journal of Biotechnology. 2008; 7:2798-2802.