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Stability of RAPD fingerprints in potato: effect of source tissue and primers

S.K. CHAKRABARTI*, D. PATTANAYAK, D. SARKAR, V.P. CHIMOTE and P.S. NAIK

Division of Crop Improvement, Central Potato Research Institute, Shimla-171001, Himachal Pradesh, India

Abstract

Variations in random amplified polymorphic DNA (RAPD) profiles from leaf, stem, root, and tuber tissues were observed in case of two glasshouse grown potato cultivars using 40 decamer primers suggesting possible danger of cultivar misidentification. Genomic DNA extracted from the above four tissues of four *in vitro* grown potato cultivars, however, produced more uniform RAPD fingerprints. A significant effect of random primers on fingerprint uniformity was observed in case of both glasshouse and *in vitro* grown samples. A new concept of stability index for random primers based on homogeneity of RAPD profiles obtained from different tissues of a single plant have been introduced. It is concluded that RAPD analysis of genomic DNA extracted from any tissue of *in vitro* grown potato plants using 14 selected decamer primers could be used to develop RAPD fingerprints for identification of Indian potato cultivars.

Additional key words: DNA amplification, molecular marker, polymerase chain reaction (PCR), *Solanum tuberosum* ssp. *tuberosum*.

Introduction

Electrophoretic comparisons of tuber proteins and enzymes had been used earlier to identify potato cultivars (Douches and Ludlam 1991). DNA-based markers have been developed during last two decades that include several polymerase chain reaction (PCR)-based techniques (Morgante and Olivieri 1993, Caetano-Anolles 1994, Zietkiewicz et al. 1994). Of these different techniques, random amplified polymorphic DNA (RAPD) is the most widely used method for DNA fingerprinting (Banerjee *et al.* 1999, Taylor and Soliman 1999, McGregor *et al.* 2000). The major drawback of RAPD genotyping arises from the lack of profile reproducibility, which is mainly due to low primer annealing temperature. Besides, several other factors like concentration of primer, template DNA and Mg^{2+} ions in the reaction mixture, and even the source of Taq polymerase strongly influence the PCR *vis-a-vis* RAPD reaction (Weising *et al.* 1995, Chakrabarti *et al.* 2001). Very little information, however, is available regarding uniformity of RAPD patterns obtained from different tissues of the same individual. It is usually assumed that similar DNA fingerprints should be obtained from any tissue of a cultivar, since the same genomic DNA is present in all somatic cells. However, it is well known that DNA preparations from different tissues of a single plant are often contaminated with specific secondary metabolites (Boiteux *et al.* 1999). It has also been reported that Taq polymerase is affected by such contaminants (Gelfand and White 1990). Therefore, in the present study we investigated the stability of RAPD profiles obtained from different tissues of a potato plant and ability of 40 random decamer primers (*Operon Technologies Inc.*, Alameda, CA, USA) to produce stable DNA fingerprints irrespective of tissue sources. Initially the experiment was conducted with two potato cultivars grown in the glasshouse. Since surface microbial contamination may be responsible for the observed fingerprint variability in case of glasshouse grown samples, an experiment was subsequently conducted with *in vitro* grown samples to rule out this possibility. The experiment with the *in vitro* samples was conducted with a different set of four cultivars since plantlets for the two cultivars tried under glasshouse condition were not available at that time. Since the objective of this

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Abbreviations: *ANOVA* - analysis of variance; CTAB - cetyltrimethylammonium bromide; EDTA - ethylenediaminetetraacetic acid; PCR - polymerase chain reaction; RAPD - random amplified polymorphic DNA; SAHN - sequential, agglomerative, hierarchical and nested clustering; UPGMA - unweighted pair group method by arithmetic average.

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* Author for correspondence; fax: (+91) 177 2624460, e-mail: skc_cpri@yahoo.co.in

experiment was to find out fingerprint stability among different tissues of same individual but not to compare fingerprint variability among individuals, it was presumed that the use of different set of cultivars for the glasshouse and *in vitro* studies would not affect the outcome of this experiment.

Materials and methods

Plants: Indexed tubers of two commercial potato (*Solanum tuberosum* L.) cultivars, Kufri Chipsona 1 and Kufri Chandramukhi, were grown in earthen pots containing 1:1:1 mixture of farmyard manure:sand:soil, supplemented with appropriate NPK fertilizers. The plants were grown in glasshouse under long day condition of summer in Shimla (latitude: 31.60N, longitude: 77.13E, altitude: 2202 msl, maximum irradiance of 2 000 μmol m^{-2} s^{-1} and temperature range 15 - 28 °C). Leaves, stems, roots and tubers of 90-d-old plants were used for extracting total genomic DNA.

Disease-free plantlets of four potato cultivars Kufri Ashoka, Kufri Bahar, Kufri Chipsona 2, and Kufri Pukhraj were maintained and multiplied *in vitro* using routine procedure (Sarkar *et al*. 1997). Plantlets were grown for 21 d under 16-h photoperiod (irradiance of 50 - 60 μmol $m^{-2}s^{-1}$) at 24 °C before extracting genomic DNA from leaves, stem and roots. Microtubers used for DNA extraction were produced *in vitro* by standard procedure (Naik and Sarkar 1998).

Template DNA preparation: Total genomic DNA from plant samples was extracted by a modified cetyltrimethylammonium bromide (CTAB) procedure (Doyle and Doyle 1987). RNA contaminants in all the samples were digested with 100 μg cm^{-3} RNase A for 30 min at 37 °C, extracted once with phenol: chloroform:isoamyl alcohol (25:24:1), precipitated by adding 0.1 volume ammonium acetate and 2 volumes of chilled ethanol, washed with 70 % ethanol and resuspended in 0.2 cm^3 sterile *MilliQ* water. Quantity and quality of DNA preparations were checked by standard spectrophotometry as well as by gel electrophoresis and all the samples were diluted to 25 μg DNA cm^{-3}.

PCR condition and temperature profile: The polymerase chain reaction was performed in a reaction volume of 0.025 cm^3 containing 1X Taq polymerase buffer with 1.5 mM $MgCl_2$ (*PE Applied Biosystem*, Foster City, CA, USA), 200 μM of each dNTP (*PE Applied Biosystem*), 25 pmol primer, 100 ng genomic DNA and 1.0 unit Taq DNA polymerase (AmpliTaq, *PE Applied Biosystem*). A total of 40 random decamer primers (*Operon Technology*, Alameda, CA, USA), known to produce good amplification products in our previous experiments (Chakrabarti et al. 1998, 2001), were used in the present study. Amplification was performed in a GeneAmp PCR System 9700 (*PE Applied Biosystems*) using 45 cycles. Each cycle consisted of 1.0 min denaturation at 94 °C, 1.0 min annealing at 35.5 °C, and 2 min extension at 72 °C. The samples were maintained at 94 °C for 5 min before the start of the first cycle and after the final cycle all amplified products were subjected to extension at 72 °C for 10 min. Amplified products were separated by electrophoresis on 1.6 % agarose gels (20 cm length) with 0.5 μg cm^{-3} ethidium bromide for 5 h at 4 V cm^{-1} constant voltage. The gels were scanned in Fluor-S™ MultiImager (*Bio-Rad Laboratories*, Hercules, CA, USA) and the images were analyzed by the Diversity Database software package (*Bio-Rad Laboratories*).

Similarity analysis and clustering: PCR amplification with each primer was performed twice by two different persons in our laboratory and the consistent bands were only considered for scoring. The amplified DNA fragments were scored for each variety as 1 (band present) or 0 (band absent) with band number 1 being the biggest fragment. Similarity matrices for glasshouse grown and *in vitro* samples were generated separately by *NTSYSpc 2.0h* computer software (Rohlf 1998) using Dice coefficient (Nei and Li 1979). The similarity matrix thus generated was used for cluster analysis by unweighted pair-group method arithmetic average (UPGMA) under SAHN (sequential, agglomerative, hierarchical, nested clustering) program of NTSYSpc. The output data was graphically represented as a phenetic tree. The goodness of fit for the dendrogram was analyzed by cophenetic correlation analysis.

Stability index of primers: Fingerprint stability index of a primer was considered as 1 if all the four tissues of a particular variety gave similar pattern, 0.75 if any three tissues gave similar pattern, 0.50 if any two tissues gave similar pattern and 0.25 if all four tissues gave different patterns. Effect of primers on stability index was analyzed by one-way *ANOVA.*

Results

RAPD-fingerprint profiles of glasshouse grown plants: Random amplification of total genomic DNA extracted from leaf, stem, root, and tuber of Kufri Chipsona 1 and Kufri Chandramukhi with 40 decamer primers produced 343 fragments, out of which 146 (43 %) were present in both the cultivars. Wide

variability was observed in RAPD profiles generated from different tissues of a single cultivar (Fig. 1). Out of 343 bands, only 88 (26 %) were stable, *i.e.* they were present in all four tissues. Among four different tissues, leaf and stem produced RAPD profiles that were more than 80 % similar. Maximum variability in RAPD profile was observed when genomic DNA from root and tuber tissues was used for PCR amplification (Fig. 1*B*). The goodness of fit of the phenogram was tested by normalized Mantel statistics using cophenetic values. Very high matrix correlation value (r = 0.92) suggested good level of association of similarity values with the phenogram. This result, therefore, emphasized danger of misidentifying samples by RAPD fingerprinting if similar tissues are not used for DNA extraction particularly in case of field or glasshouse grown samples.

Fingerprint uniformity of *in vitro* sample: Random amplification of genomic DNA extracted from leaf, stem, root and microtuber tissues of four *in vitro* grown potato cultivars with the same 40 decamer primers produced 383 fragments out of which 92 (24 %) were present in all four cultivars (monomorphic). In addition, these 92 fragments were also found to be stable, *i.e.*, they were present in all four tissues. RAPD profiles generated by using the random primer OPD 03 are shown in Fig. 2*A*. Similarity of fingerprints among different tissues of a particular cultivar was > 90 % (Fig. 2*B*). This suggested that RAPD fingerprints generated from genomic DNA of any *in vitro* tissue of a potato cultivar could be used for its identification with 90 % accuracy.

Effect of primers on fingerprints uniformity: Fingerprint uniformity within four different tissues of a particular cultivar produced by different primers was analyzed by one-way *ANOVA* of stability indices. Significant effect of primers on fingerprint uniformity was observed in case of glasshouse grown (calculated F = 4.875), *in vitro* (F = 4.701) as well as pooled samples (F = 1.646). None of the 40 random primers could produce uniform RAPD profiles from leaf, stem, root and tuber tissues of the two glasshouse grown cultivars. On the contrary, 14 out of 40 primers produced uniform fingerprints from all four tissues of *in vitro* samples (Table 1), even though a significant effect of primers on fingerprint uniformity was observed. This observation suggested that prior screening of random primers is necessary in order to select those giving uniform profiles irrespective of template DNA source. The effect of primer was more prominent in case of *ex vitro* samples since it was aggravated by combined effect of extraneous contaminations.

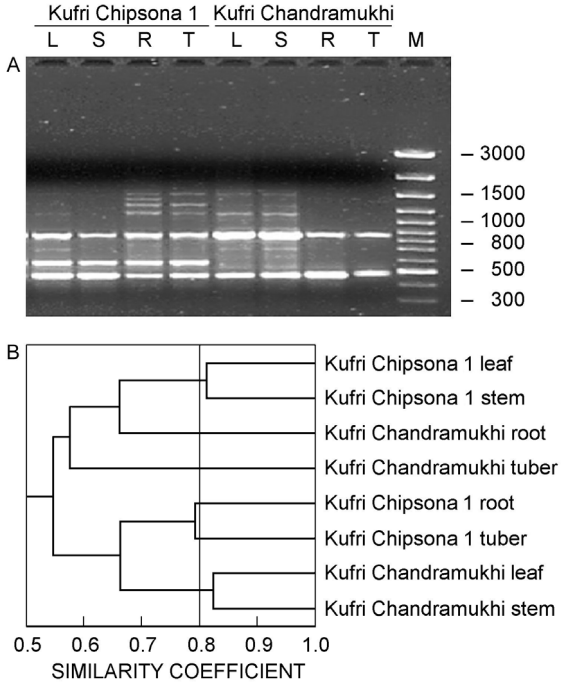

Fig. 1. *A*: RAPD profiles of glasshouse grown Kufri Chipsona 1 and Kufri Chandramukhi generated using the random primer OPA 17 from leaf (L), stem (S), root (R) and tuber (T) tissues. M denotes 100 bp DNA stepladder; *B*: dendrogram showing relationship between leaf, stem root and tuber of Kufri Chipsona 1 and Kufri Chandramukhi on the basis RAPD data.

Discussion

RAPD-based fingerprinting has been used extensively for identification of cultivars in barley (Reddy and Soliman 1997), chickpea (Banerjee *et al.* 1999), pea (Samec *et al.* 1998), soybean (Taylor and Soliman 1999) and many other crops. In case of potato, RAPD markers have been used for characterization of potato cultivars in North America (Sosinski and Douches 1996), Russia (Organisyan *et al.* 1996), Japan (Hosaka *et al.* 1994), Australia (Ford and Taylor 1997), Canada (Demeke *et. al.* 1993), India (Chakrabarti *et al.* 1998, Pattanayak *et al.* 2002). However, in majority of such instances, only one type of tissue (either leaf, sprout or tubers) has been used for DNA extraction. Our result, however, suggested that RAPD profile obtained from one type of tissue might be different from that of others of the same potato cultivar. To our knowledge, influence of random primers on uniformity of RAPD fingerprints developed from different tissues of a particular plant cultivar has not been reported elsewhere. This problem is more serious in case of glasshouse or field grown samples. Surface contamination of aerial and underground plant parts with different micro-flora and -fauna might be an important factor contributing to fingerprint variability. Genomic DNA from surface microorganisms would also be extracted along with that from plant tissue. Extreme sensitivity of PCR technique would co-amplify such contaminated DNA along with plant DNA. In fact it has been demonstrated that leaf tissue from cucumber plants

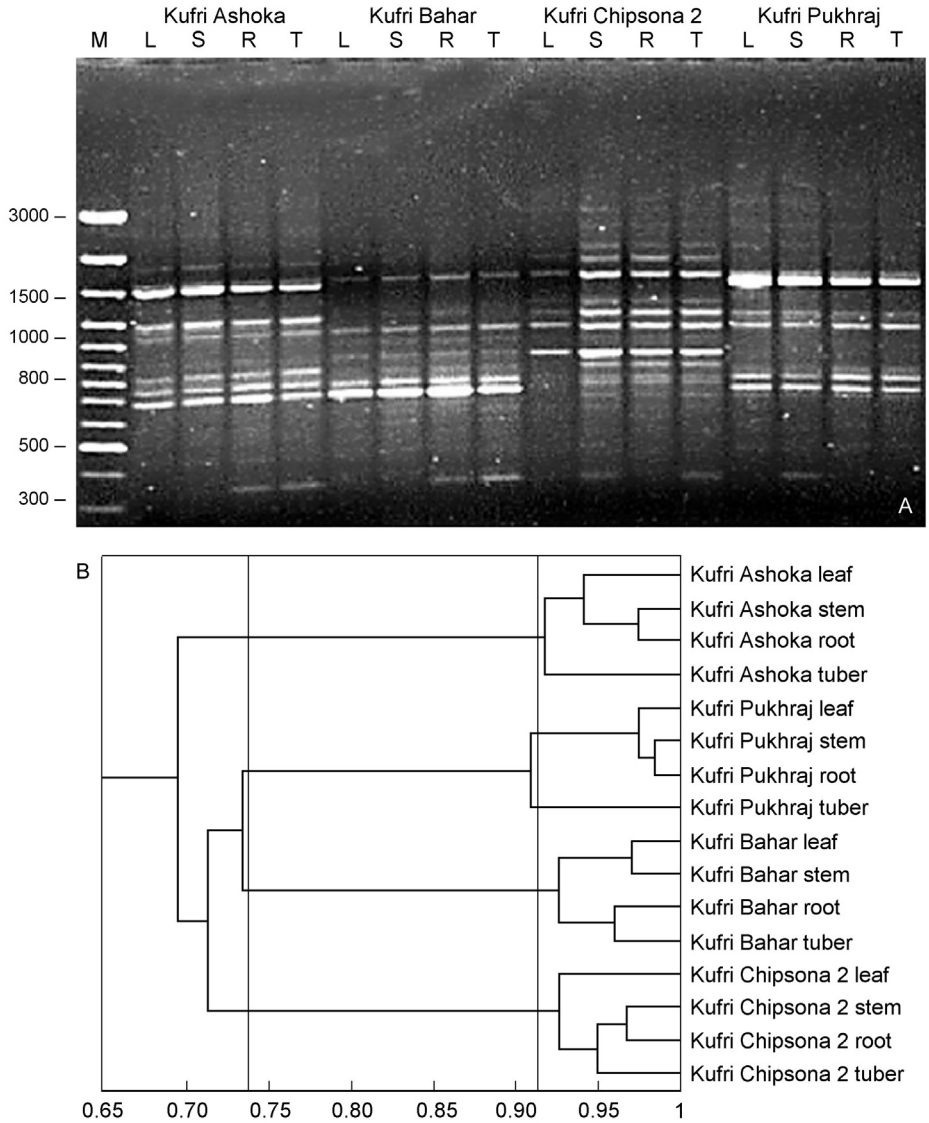

Fig. 2. *A*: RAPD profiles generated from leaf (L), stem (S), root (R) and tuber (T) tissues of four *in vitro* grown potato cultivars, Kufri Ashoka, Kufri Bahar, Kufri Chipsona 2 and Kufri Pukhraj using the random primer OPD 03. M denotes 100 bp DNA stepladder; *B*: dendrogram showing relationship between leaf, stem root and tuber of Kufri Ashoka, Kufri Bahar, Kufri Chipsona 2 and Kufri Pukhraj on the basis RAPD data.

infected with *Sphaerotheca fuliginea* showed variation in RAPD banding patterns compared with those of healthy plants (Staub *et al.* 1996). Similar RAPD profiles obtained either from tuber/root or leaf/stem tissues of a particular potato cultivar in our experiment also indicated that contamination of such pairs of tissue samples with similar type of microflora might be a contributing factor for profile variability. Differential accumulation of polyphenols, sugars, proteases, *etc*., in different plant organs during normal growth in fields and glasshouse might be another important factor contributing to profile instability (Boiteux *et al.* 1999). Tissue specific differences in isozyme banding patterns have been reported earlier in many plant species (Higgins 1984). Our observation suggested that it is also true for RAPD markers at least under glasshouse/field condition for potato. Similar observation has been reported in case of cucumber (Staub *et al.* 1996) and carrot (Boiteux *et al.* 1999). On the contrary, no effect of plant tissue had been observed in case of apple (Autio *et al.* 1998), American elm (Kamalay and Carey 1995), and Norway spruce (Skov 1998).

Absence of microbial contamination and relatively lower secondary metabolites in *in vitro* grown plants were mainly responsible for uniformity in RAPD profiles obtained from different tissues. Our result, therefore, suggested that any tissue from *in vitro* grown samples could be used for fingerprint comparison using pre-

Table 1. Random primers producing uniform RAPD patterns of genomic DNA isolated from leaf, stem, root, and tuber of four *in vitro* grown potato cultivars.

No.	Primer	Sequence (5'-3')	Fragments amplified	Size range [bp]
1	OPA 04	AAT CGG GCT G	11	452-2473
2	OPA 06	GGT CCC TGA C	4	856-2925
3	OPA 17	GAC CGC TTG T	12	490-1552
4	OPB 05	TGC GCC CTT C	10	677-2282
5	OPB 07	GGT GAC GCA G	11	427-2289
6	OPB 08	GTC CAC ACG G	12	208-3027
7	OPB 09	TGG GGG ACT C	4	867-1707
8	OPB 10	CTG CTG GGA C	9	526-2449
9	OPB 11	GTA GAC CCG T	2	720- 849
10	OPB 15	GGA GGG TGT T	8	319- 916
11	OPC 08	TGG ACC GGT G	9	375-1415
12	OPC 12	TGT CAT CCC C	11	434-2967
13	OPC 19	GTT GCC AGC C	9	547-2391
14	OPD 05	TGA GCG GAC A	9	291-2168

selected primers. Repeated transfer during micropropagation and long-term conservation has been shown to cause genetic changes in several plant species that might be responsible for producing different RAPD profiles from that of original field grown sample (Piccioni *et al*. 1997). However, in case of potato no such changes in RAPD profile has been noticed even after long term storage with ancymidol (Sarkar *et al*. 2001). Though epigenetic changes due to micropropagation have been reported in potato, such changes have not been found to affect RAPD fingerprints (Joyce *et al*. 1999).

It is, therefore, concluded that identification of potato cultivars based on RAPD fingerprints developed from field or glasshouse grown plants would be erroneous. More robust technique like simple sequence repeat (SSR) based DNA fingerprints should be used in case of potato (Chimote *et al*. 2004). If RAPD has to be used for fingerprint development in potato, *in vitro* grown plantlets should be used for genomic DNA extraction.

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