MOLECULAR MARKERS FOR GENETIC STABILITY OF INTERGENERIC HYBRIDS *Fragaria* x *Potentilla* DERIVED FROM TISSUE CULTURE

Anca Nicoleta SUTAN[*], Aurel POPESCU[*], Raluca GHEORGHE[**], Carmen Florentina POPESCU[**], Valentina ISAC[***]

[*] *University of Pitesti, Faculty of Sciences, Department of Biology and Horticulture, Pitesti, Romania*
[**] *National Research and Development Institute for Biotechnology in Horticulture, Laboratory of Molecular Genetics, Stefanesti, Romania*
[***] *Research Institute for Fruit Growing, Pitesti, Romania*
Corresponding author: Anca Nicoleta Sutan, University of Pitesti, Faculty of Sciences, Department of Biology and Horticulture, 1 Targu din Vale, 110040 Pitesti, Romania, tel.: 0040348453100, fax: 0040348453123, e-mail: ancasutan@yahoo.com

Abstract. The effect of growth regulators, explant source and culture age on genetic stability of plants obtained from tissue culture propagation of ornamental strawberry “Serenata” were examined. Genomic DNAs of *in vitro*-derived shoots and control plant were extracted and compared by RAPD-PCR analyses. Ten primers (from 48 previously tested) were selected and used in RAPD analysis to prove the clonal fidelity (i.e. genetic stability) of the tissue culture-derived ornamental strawberry plants. The lack of polymorphisms in micropropagated plants screened through molecular markers was used to suggest genetic fidelity. Identicaly banding patterns of the RAPD profiles obtaining from vitroplants, regenerated via organogenesis or meristems culture, suggested that in the ornamental strawberry, variety “Serenata”, neither explant source, nor callus age or limited number of subcultures, in basal media supplemented with low concentration of growth regulators, were associated with occurence of somaclonal variation.

Keywords: tissue culture, intergeneric hybrids, RAPDs, clonal fidelity

INTRODUCTION

In recent years, with the advent of recombinant DNA technology and PCR, molecular markers are being used for a variety of studies. The molecular markers, including RAPDs, have also been used for testing the genetic fidelity of micropropagated and organogenesis derived plants or elite genotypes. Nevertheless, abnormalities were observed in some cases of tissue-culture strawberry plants, when compared to conventionally propagated ones [11, 12, 14, 22, 30].

Although *in vitro* culture techniques have monopolized mass propagation and are of beneficial use in various fields, in plant breeding [31], the morphogenesis seems to be highly dependent on plant growth regulators [1], media used for culture [21, 27], or explant source [9, 22, 27], wich are closely related with the genotype [14, 28]. In this context, the risck of genetic variations, induced by tissue culture, and the usefulness and suitability of RAPD markers, for assessing the genetic stability of micropropagated strawberry [6, 24], must be emphasized.

The economic consequences of somaclonal variation, can be enormous, so it is extremely important to ascertain the suitability of a particular micropropagation protocol developed for a particular species, in terms of the production of genetically identical plants [29].

Analysis of genomic DNA is commonly used in tissue culture-derived plants, allowing the characterization of genotypes, regardless of plant physiological stage or environmental conditions [18]. Furthermore, the time required for the provision of highly informative results are short. Several cytological, isozyme and molecular markers have been used to detect the variation and/or confirm the genetic stability in micropropagated strawberry plants [4, 5, 30, 33]. In different plant species, the use of PCR, especially in the form of RAPDs [3], has proved to be useful for identification of genetic variation, depending only by the number of the primers involved.

In the present study, the genetic stability and uniformity of shoots obtained in six different tissue culture conditions were assessed by using RAPD markers.

MATERIALS AND METHODS

Plant material: An intergeneric hybrid of *Fragaria x Potentilla*, named “Serenata”, from the *Fragaria* Germplasm Collection of the Research Institute for Fruit Growing, Pitesti, Mărăcineni, Romania, it was investigated. In order to determine the genetic fidelity of shoots formed by different tissue-culture methods, this variety of ornamental strawberry was established *in vitro* culture starting from meristems, leaf and petiole pieces (Table 1).

In this study, RAPD technique was employed, as it has the advantage of simplicity, is quickly performed and is of relatively low cost. Also, it requires very little plant material and quick DNA extraction protocols are suitable.

DNA extraction: Total genomic DNA was extracted from both tissue culture-derived plants and field grown mother plant using DNEasy Plant Mini Kit (Qiagen), following the manufacturers protocol.

To check the degradation of DNA, samples were loaded into 0.8% agarose gel in 0.5 × TBE submitted to electrophoresis, stained with ethidium bromide and observed under ultraviolet (UV). The DNA used for the amplification was of high molecular weight, with little degradation and free of RNA, as revealed by UV and gel electrophoresis.

PCR conditions: RAPD amplification was performed in a reaction volume of 25 μl containing 4 mM $MgCl_2$, 0.4 mM of each dNTP, 0.6 units Taq DNA polymerase, 0.8 μM primer and 100-200 ng genomic DNA. The amplification reactions were carried out in an TC-512 Gradient Thermocycler (Bibby Scientific Ltd) programmed as following: preliminary denaturation of DNA at 95°C for 2 min, 45 cycles of 92°C for 30 s, 36°C for 25 s and 72°C for 74 s, and a final extension step at 72°C for 7 min. The PCR pro-

Table 1. The origin of tissue culture regenerated shoots in the intergeneric hybrid *Fragaria x Potentilla* tested for genetic fidelity.

No.	Intergeneric hybrid of *Fragaria x Potentilla*	Basic culture medium and combinations of growth regulators	Tissue-culture methods	Plant material provenance
1.	"Serenata"	- Lee & Fossard [19] - 1.0mg/l BAP, - 1.0 mg/l IAA, - 0.1 mg/l GA_3	Micropropagation (starting from meristems with 2-3 leaf primordia, 0.1-0.3 mm in size)	Fourth subculture (120 days of *in vitro* culture)
2.	"Serenata"	- Murashige & Skoog [23] - 1.0mg/l BAP, - 1.0 mg/l IAA, - 0.1 mg/l GA_3	Micropropagation (starting from meristems with 2-3 leaf primordia, 0.1-0.3 mm in size)	Fourth subculture (120 days of *in vitro* culture)
3.	"Serenata"	- Knop [16] - 1.0mg/l BAP, - 1.0 mg/l IAA, - 0.1 mg/l GA_3	Micropropagation (starting from meristems with 2-3 leaf primordia, 0.1-0.3 mm in size)	Fourth subculture (120 days of *in vitro* culture)
4.	"Serenata"	- Lee & Fossard [19] - 0.5 mg/l IBA, - 3.0 mg/l BAP	Organogenesis (starting from leaf explants)	70 days of *in vitro* culture (3-4 weeks of callus phase)
5.	"Serenata"	- Lee & Fossard [19] - 0.5 mg/l IBA, - 3.0 mg/l BAP	Organogenesis (starting from petiole explants)	70 days of *in vitro* culture (3-4 weeks of callus phase)
6.	"Serenata"	- Murashige & Skoog [23] - 1.0 mg/l IBA, - 3.0 mg/l BAP	Organogenesis (starting from leaf explants)	70 days of *in vitro* culture (3-4 weeks of callus phase)
7.	"Serenata" - Control plant	N/A	N/A	Germplasm collection

ducts were separated by gel electrophoresis on a 1.2 % agarose gel, in 0.5 x TBE buffer during 1 h at 90 V and stained with ethidium bromide (10 mg/ml).

A total of 48 primers were screened initially for amplification capacity with the template DNA. Ten RAPD markers were finally selected for analysis on the basis of their amplification products for clear, bright and scorable banding patterns.

RAPD analysis using each primer was repeated at least twice, in order to establish the reproducibility of banding pattern of the DNA sample studied. Electrophoretic DNA bands of low visual intensity that could not be readily distinguished as present or absent were considered ambiguous markers and were not scored.

Photographs of the gels were obtained with a Gene Flash Syngene Bio Imaging. The 100 bp DNA Ladder (Fermentas) was used as a molecular size standard and the size of the amplification products was estimated using LabImage software from the photographs of the gels.

RESULTS

On the basis of the number, intensity and reproductibility of RAPD bands, ten random primers (Table 2) were selected out of the 48 previously tested and used in RAPD analysis to prove the clonal fidelity (i.e. genetic stability) of the tissue culture-derived strawberry plants.

Table 2. List of primers, their sequence and size of the amplified fragments generated by 10 RAPD primers.

No.	Primer	Sequence	No of scorable bands	No of monomorphic bands	Size in bp
1	OPA02	TGCCGAGCTG	5	5	2248-810
2	OPA11	CAATCGCCGT	10	10	2667-312
3	OPA19	CCAACGTCGG	5	5	2356-1138
4	OPA20	GTTGCGATCC	5	5	1881-986
5	OPB09	TGGGGGACTC	4	4	1713-710
6	OPB10	CTGCTGGGAC	10	10	2600-480
7	OPB15	GGAGGGTGTG	5	5	2351-973
8	OPC05	GATGACCGCC	4	4	1755-750
9	OPC06	GAACGGACTC	5	5	2140-598
10	OPC09	CTCACCGTCC	7	7	2774-647

The primers selected for this study gave rise to a total of 60 scorable bands ranging from 312 bp to 2774 bp in size. From the selected markers, two (OPA11 and OPB10) yielded 20 bands across the shoots tested, and another one (OPC09) yielded 7 bands. The other primers were not as efficient in generating succesive PCR products. A total of 420 bands (number of plantlets analyzed X number of bands with all primers) were generated by the RAPD technique, giving rise to monomorphic patterns across all plantlets studied. All RAPD profiles from the tissue culture-derived ornamental strawberry plants were found to be analogous to those of the control plant, indicating either identity or similarity. Examples of RAPD patterns amplified with primers OPC09 and OPB09 are shown in Fig. 1 A & B.

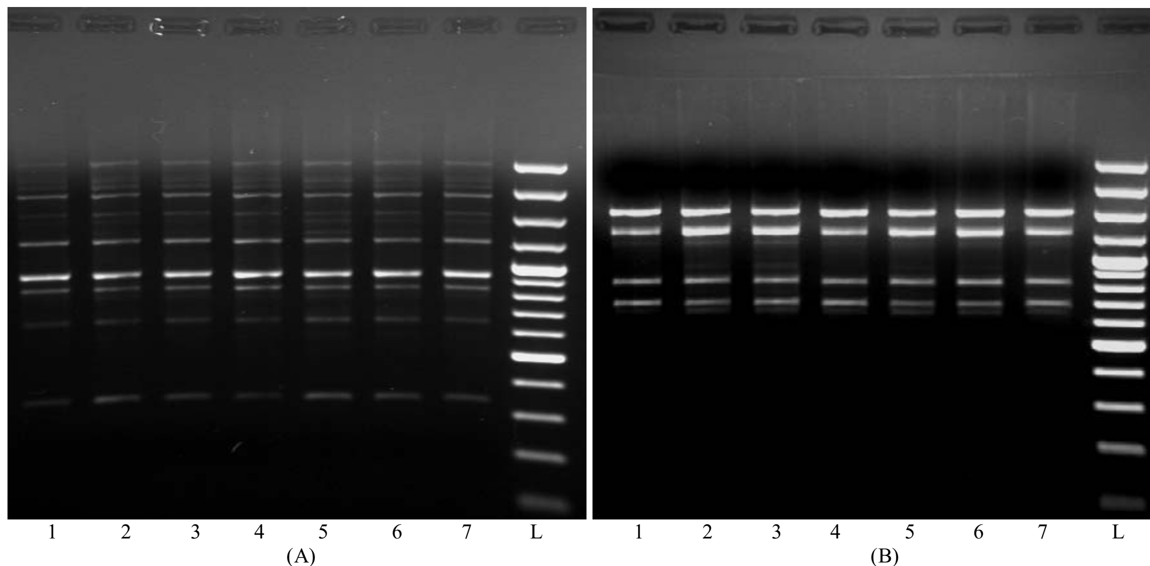

Figure 1. RAPD profiles of the plants investigated with primers OPC09 (a) and OPB09 (b). The numbers represent the plant material investigated in this study, as presented in Table 1; L= 100 bp DNA ladder

Identicaly banding patterns of the RAPD profiles obtaining from vitroplants, regenerated via direct organogenesis, and from control plant, suggested that in the ornamental strawberry, variety "Serenata", neither explant source, nor callus age were associated with occurence of somaclonal variation. Also, the correspondence between RAPD profiles of control plant and microshoots of intergeneric hybrids "Serenata", after four subcultures, in basal media supplemented with low concentration of growth regulators, denote that no genetic variation had occurred.

DISCUSSIONS

In this study, using 10 RAPD primers, no polymorphism was detected among the shoots regenerated starting from meristems or leaf discs. This results were similar to those of Nehra *et al.* [26] who found no variants for plants derived from meristem culture or direct leaf regeneration for strawberry cultivars "Vecstar" and "Redcoat". Among the various methods developed to micropropagate strawberry, enhanced axillary branching culture has become the most important, especially because it is generally considered to be an *in vitro* culture sistem with low risc of genetic instability [17, 20]. However, the occurrence of somaclonal variation should not be overruled.

Most somaclonal variations occur in plants regenerated from *in vitro* cultures that have undergone a dedifferentiation phase [17]. Also, high frequency of somaclonal variation was obtained among plants regenerated from 16 and 24-week-old-calli [25] or multiplicated on basal media supplemented with high concentration of growth regulators, such as BAP and 2,4-D [25, 10].

The DNA amplification products, could result from changes in either the sequence of the primer binding sites or changes which alter the size and present the successful amplification of target DNA. In the present study, the amplifies products exhibited monomorphism among micropropagated intergeneric hybrids *Fragaria x Potentilla*, irrespective of culture media, and were similar to those of the control plant. Sporadic occurences of somaclonal variations were observed in some cultivars of strawberry, always after a high number of subcultures [4, 13]; therefore it is suggested to limit the number of transfers to 5-10 times.

Because there were no changes in the banding patterns observed in the tissue culture plants as compared with that of the control plant, we conclude that our micropropagation protocol can be followed without much risk of genetic instability. Furthermore, the lack of polymorphic bands among the RAPD profiles of microshoots regenerated through organogenesis, can be associated with a higher genetic stability of this genotype. Other studies have also shown that the genotype of plant can affect the amount of variability that occurs as a consequence of culturing tissues *in vitro* [8].

The results obtained in this study confirmed the usefulness and applicability of the RAPD method in determining genetic stability and uniformity of plants clonally propagated *in vitro*. The clonal fidelity of *in vitro* regenerated shoots has also been determined with the RAPD tehnique in other species, such as *Begonia* sp. [2], *Lilium* sp. [32], *Drosera* sp., [15], *Dendrobium* hybrid [7]. The informative primers identified and tested in our studies will be useful in genetic analysis of ornamental strawberry varieties in germplasm holdings.

REFERENCES

[1] Biswas, M.K., Dutt, M., Roy, U.K., Islam, R., Hossain, M., (2009): Development and evaluation of *in vitro* somaclonal variation in strawberry for improved horticultural traits. Scientia Horticulturae, 122(3): 409-416.

[2] Bouman, H., De Klerk, G-J., (2001): Measurement of the extent of somaclonal variation in begonia plants regenerated under various conditions.Comparison of three assays. Theoretical and Applied Genetics, 102: 111–117.

[3] Brown, P.T.H., Lange, F.D., Kranz, E., Lorz, H., (1993): Analysis of single protoplasts and regenerants plants by PCR and RAPD technology. Molecular and General Genetics, 237: 311-317.

[4] Boxus, P.H., Jemmali, A., Terzi, J.M., Arezki, O., (2000): Drift in genetic stability in micropropagation: The case of strawberry. Acta Horticulturae, 530: 155-162.

[5] Damiano, C., Ascarelli, A., Frattarelli, A., Lauri, P., (1995): Adventitious regeneration and genetic variability in strawberry. Acta Horticulturae, 392: 107-114.

[6] Degani, C., Rowland, L.J., Saunders, J.A., Hokason, S.C., Ogden, E.L., Golan-Goldhirish, A., Galetta, G., (2001): A comparison of genetic relationship measures in strawberry (*Fragaria x ananassa* Duch.) based on AFLPs, RAPDs, and pedigree data. Euphytica, 117: 1-12.

[7] Ferreira, W.M., Kerbauy, G.B., Costa A.P.P., (2006): Micropropagation and genetic stability of a *Dendrobium* hybrid (*Orchidaceae*). In Vitro Cellular & Developmental Bioloy - Plant, 42(6): 568-571.

[8] Hammerschlag, F., Garces, S., Koch-Dean, M., Ray,S., Lewers, K., , Maas, J., Smith, J.B., (2006): *In vitro* response of strawberry cultivars and regenerants to *Colletotrichum acutatum*. Plant Cell, Tissue and Organ Culture, 84: 255-261.

[9] Hao, Y.J., Deng, X.X., (2002): Analysis of ploidy and the patterns of amplified fragment length polymorphism and methylation sensitive amplified polymorphism in strawberry plants recovered from cryopreservation. Cryoletters, 23: 37-46.

[10] Irkaeva, N.M., Matveeva, T.V., (1997): Responce of strawberry (*Fragaria vesca* L.) strain to cytokinin *in vitro*. Genetika, 33: 495-500.

[11] Jemmali, A., Boxus, Ph., Dekegel, D., Van Heule, G., (1994a): Occurrence of spontaneous shoot regeneration on leaf stipules in relation to hyperflowering response in micropropagated strawberry plantlets *in vitro*. Cell & Devevelopmental Biology, 30: 192-195.

[12] Jemmali, A., Boxus, Ph., Kevers, C., Gaspar, Th., (1994b): Flowering abundance of strawberry depending on the number of subcultures *in vitro*. Relationships with growth, rooting, peroxidase activity. pp. 356-362. In: Lumsden P.J. et al. (ed.), Physiology, Growth and Development of Plants in Culture. Kluwer Academic Publishers, Dordrecht.

[13] Karp, A., (1995): Somaclonal variation as a tool for crop improvement. pp: 295-303. In: Cassell, A.C., Jones, P.W. (ed.), Plant Genetic Manipulation: Criteria for Decision Making. Kluwer Academic Publishers, Dordrecht.

[14] Kaushal, K., Nath, A.K., Kaundal, P., Sharma, D.R., (2002): Studies on somaclonal variation in strawberry (*Fragaria x ananassa* Duch.) cultivars. Acta Horticulturae, 662: 269-275.

[15] Kawiak, A., Łojkowska, E., (2004): Application of RAPD in the determination of genetic fidelity in micropropagated *Drosera* plantlets. In Vitro Cellular & Developmental Biology*,* 40: 592-595.

[16] Knop, W., (1965): Quantitative Untersuchungen Über den Ernährungensprozess der Pflanze. Landwisrtsch. Versuch-St, 7: 93-107.

[17] Kumar, M.K., Barker, R.E., Reed, B.M., (1999): Morphological and molecular analysis of genetic stability in micropropagated *Fragaria* x *ananassa* cv. Pocahontas. In Vitro Cellular & Developmental Biology, 35: 254-258.

[18] Kuras, A., Korbin, M., Zurawicz, E., (2004): Comparison of suitability of RAPD and ISSR techniques for determination of strawberry (*Fragaria x ananassa* Duch.) relationship. Plant Cell, Tissue and Organ Culture, 79: 189-193.

[19] Lee, E.C.M., de Fossard, R.A., (1977): Some factors affecting multiple bud formation of strawberry (*Fragaria x ananassa* Duch.) *in vitro*. Acta Horticulturae, 78: 187-196.

[20] Mohamed, A.E., (2007): Somaclonal variation in micropropagated strawberry detected at the molecular level. International Journal of Agriculture & Biology, 9(5): 721-725.

[21] Morere-Le Paven, M.C., De Buyser, J., Henry, Y., Corre, F., Hartmann, C., Rode, A., (1992): Multiple pattern of mtDNA reorganization in plants regenerated from different *in vitro* cultured explants of a single wheat variety. Theoretical and Applied Genetics, 85: 9–14.

[22] Morozova, T., (2003): Genetic stability of pure line of *Fragaria vesca* L. in micropropagated and long term storage *in vitro*. Acta Horticulturae, 567: 85–88.

[23] Murashige, T., Skoog, F., (1962): A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiologia Plantarum, 15(3): 473-497.

[24] Milella, L., Saluzzi, D., Lapelosa, M., Bertino, G., Spada, P., Greco, I., Martelli, G., (2006): Relationships between an Italian strawberry ecotype and its ancestor using RAPD markers. Genetic Resources and Crop Evolution, 53: 1715–1720.

[25] Nehra, N. S., Kartha, K. K., Stushnoff, C., Giles, K. L., (1992): The influence of plant growth regulator concentrations and callus age on somaclonal variation in callus culture regenerants of strawberry. Plant Cell, Tissue and Organ Culture, 29:257-268.

[26] Nehra, N. S., Kartha, K. K., Stushnoff, C., Giles, K. L., (1994): Effect of *in vitro* propagation methods on field performance of two strawberry cultivars. Euphytica, 76:107-115.

[27] Palombi, M.A., Monticelli, S., Festa, A., Damiano, C., (2003): In vitro adventitious regeneration affects the genetic stability of strawberry plants (*Fragaria x ananassa* Duch.). Acta Horticulturae, 616: 459-462.

[28] Popescu, A.N., Isac, V.S., Coman, M.S., Radulescu, M., (1997): Somaclonal variation in plants regenerated by organogenesis from callus culture of strawberry (*Fragaria x ananassa*). Acta Horticulturae, 439: 89-96.

[29] Rani, V., Raina, S.N., (2000): Genetic fidelity of organized meristem-derived micropropagated plants: a critical repraisal. In Vitro Cellular & Developmental Biology - Plant, 36: 319-330.

[30] Sansavini, S., Rosati, P., Gaggioli, D., Toshi, M.F., (1990): Inheritance and stability of somaclonal variation in micropropagated strawberry. Acta Horticulturae, 280: 375-84.

[31] Taji, A., Kumar, P.P., Lakshmanan, P., (2002): In Vitro Plant Breeding. Food Products Press, New York, p. 167.

[32] Varshney, A., Lakshmikumaran, M., Srivastava, P.S., Dhawan,V., (2001): Establishment of genetic fidelity of *in vitro*-raised *Lilium* bulbets through RAPD markers. In Vitro Cellular & Developmental Bioloy - Plant, 37: 227–231.

[33] Williams, J.G.K, Kubelik, A.R., Livark, K., Rafalski, A., Tingey, S.V., (1990): DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Ressearch, 18: 6531-6535.