Pak. J. Bot., 33(4): 411-417, 2001.

EVALUATION OF GENETIC STABILITY IN THE BLACKCURRANT PLANTS REGENERATED VIA MICROPROPAGATION USING RAPD-PCR TECHNIQUE

SAIFULLAH KHAN* AND WILLIAM SPOOR

School of Agriculture, University of Edinburgh, Edinburgh, U.K.

Abstract

Blackcurrant (*Ribes nigrum* L.) cultivar Ben Sark was multiplied using meristem culture up to 16 generations. The leaves from each generation/subculture were collected and used to isolate DNA. RAPD-PCR technique was used to evaluate the genetic purity of the regenerated plants. The DNA profile of parent explant source and 16 regenerated plants were screened using 12 ten base random primers. Only reproducible fragments with intense bands were scored which generated 77 DNA fragments, thus 1540 bases were explored in this set of experiment. All the regenerated plants upto 14 subculture showed 100% similarity. In subculture 15 and 16, the similarity were 93.7 and 86% with variation 6.2 and 13.4, respectively. Production of variant plants from meristem culture is not good for commercial propagation. However, it could be minimised by reducing the subculture cycle. Additionally, this variability could be used for further improvement and selection of new cultivars and an important source of variability be exploited.

Introduction

Blackcurrant (*Ribes nigrum* L.) are traditionally propagated by hard wood cuttings of 20 cm in length which are directly planted in the field in the autumn season or early winter (Anon., 1981). With the introduction of new cultivars and limited supply of virus-free clones of existing cultivars, there has been a need for a method of propagation that is more economical in the use of plant materials (Wainwright *et al.*, 1986). The potential of micropropagation was explored and found to be very successful by several workers. However, the genetic integrity of regenerated plants via micropropagation was assumed rather than tested rigidly. It has been established now that tissue culture regimes may have an effect on the genetic constitution of plants. Therefore, there is a need to assess the genetic integrity of micropropagated plants in order to have more knowledge about the genetic stability of regenerated plants.

Materials and Methods

Plant material

Plant material of blackcurrant cultivar Ben Sark at the first sub-culture stage was supplied by the Department of Horticulture, Scottish Agriculture College, Bush Estate, and were subcultured in the M S medium supplemented by 30 g/l sucrose and 1.4 mg/l of the hormone BAP. Three meristem shoots were placed, under aseptic conditions, into

*Present address. In-charge Plant Tissue Culture and Biotechnology Div., International Centre for Chemical Sciences, H.E.J. Research Institute, University of Karachi, Karachi, Pakistan.

each culture jar. These were incubated in the tissue culture room (26°C ± 1°C) for a four week period, after which these plants were further sub-cultured and at this time three samples of leaves were collected by pooling within each bunch of plantlets originating from the single shoot pieces. These leaf samples were used for fresh DNA extraction in replicated runs. Samples were collected only from those plantlets which were further subcultured to store at -80°C for analysis. Material was sub-cultured every four week and leaf samples taken for 16 generations.

DNA extraction

Total genomic DNA was isolated from the frozen leaf material stored at -80°C using a modification of CTAB procedure (Doyle & Doyle, 1987). To confirm the successful extraction, the extracted DNA was visualised after electrophoresis in 1.4% agarose gel and subsequently stained in ethidium bromide. Extracted DNA showed a single sharp band of greater than 23k bp. DNA of any sample showing smearing or the absence of sharp bands was discarded and the extraction was repeated. After successful extraction, the DNA samples were quantified by using spectrophotometry. All samples were diluted accordingly to make the final concentration of 10 ng/µl. Four µl of the DNA solution was used in each 50 µl (total volume of PCR reaction mixture), as template DNA. The protocol of Williams *et al.*, (1990) was used. In each PCR, DNA from parent mother plant from which the meristem was taken to initiate the micropropagation was also included and each sample was compared with the parent plant for differences. Each reaction was repeated twice with the same conditions with fresh DNA extraction and only the reproducible bands were included in the analysis. Weak bands were not scored because they were rarely reproducible to ensure the reliability of the assay. A negative control without target DNA was always run in each PCR amplification to detect any contamination in the reaction mixture.

Primer screening

A total of 45 primers were screened. Primers were selected on the principle that primers able to amplify more DNA fragments gave more information. Therefore, primers were tested using the DNA from the parent plant as template. The number of random fragments amplified by each primer was counted and primers not showing satisfactory amplification were discarded from the programme. The primers showing many bands with good resolution and separation were selected for use. A total of 12 primers produced good amplifications and were included in this study (Table1).

Data analysis

Amplification products of each DNA fragment were represented by a band on a photograph. Each band represented a set of DNA segments/fragments of almost equal length. (The words segment, fragment and band have been used synonymously, representing a single set of DNA segments of equal length). The number of bands produced by each primer for each sample was scored. The total number of bands produced by all the primers used for a single sample was counted and compared with the total number of bands produced by the same primer for the parent.

Table 1. Primers and their base sequence used for the detection of polymorphism in regenerants of blackcurrant cultivar Ben Sark.

No.	Printer	Sequence
1	87729801	5' CACTGCAGTC 3'
2	87729804	5' TTCAGGGGTC 3'
3	87729805	5' GGACTAGTGG 3'
4	87729806	5' CCAATCCGTG 3'
5	87729807	5' GGAGCTTGAC 3'
6	87729808	5' GGAGCTTGCC 3'
7	87729809	5' CGGTGAGGCT 3'
8	87729810	5' TCACGGCACC 3'
9	87729811	5' TGCCAGTGGA 3'
10	PO298	5' CAGTTCGAGG 3'
11	OPH-01	5' GGTCGGAGAA 3'
12	OPH-03	5' AGACGTCCAC 3'

Genetic similarity estimate

Data generated from detection of the fragments were analysed employing the equation of Nei & Li (1979). Each of the clear, RAPD bands across all the samples was assigned a number (1, 2, 3, 4....n). Each band was treated as a unit character and the samples were scored for the presence or absence of a band and coded as 1 or 0, respectively. Genetic similarity was calculated between the parent and regenerated plant / tissue cultured material. This is referred to as "shared fragments", this coefficient is defined as the number of shared bands in the two samples divided by the total number of bands produced by two samples (Nei & Li, 1979). The similarity between samples was used to generate a matrix, according to the following formula:

$$\text{Similarity} = (2 \times N_{ab}) / (N_a + N_b)$$

where

N_{ab} = number of shared fragments between individual "a" and "b"

N_a = number of scored fragments of individual "a" and

N_b = number of scored fragments of individual "b"

Because similarity was estimated between different samples and the parent plant , therefore sample "a" in all cases was parent plant similarity between parents and individual sample. Variation between parents and each sample was calculated by the equation as:

$$\text{Variation (\%)} = (1\text{-Similarity}) \times 100$$

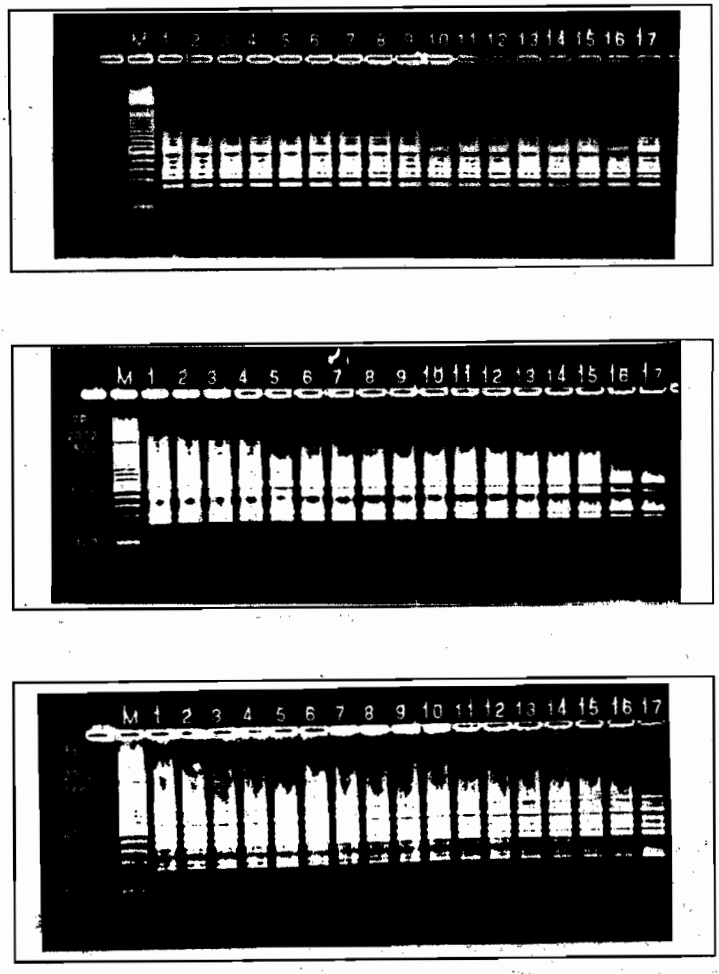

Fig. 1. A,B and C. Amplification products derived from micropropagation of 16 subculture/ generation cycle of blackcurrant cultivar Ben Sark using random 10 base primers 1, 2, and 3 respectively. From left to right: M represent nucleic acid marker (Fragment size of the DNA marker are given in base pairs). The DNA samples consists of No. 1 parent DNA, those from 2-17 are successive subcultures.

Results

Twelve primers (Table 1) were used to screen the 16 subcultures of the blackcurrant cultivar Ben Sark. All primers used in the analysis gave between 3 to 9 DNA bands with an average of 6.4 band per primer. A total of 77 DNA fragments were amplified which means 1540 bases were explored in this experiment. All DNA fragments produced were monomorphic as shown in Figures 1, 2, and 3 showing 100% similarity to the parent and with each other except the sub-culture number 15 and 16 as shown in the Table 2. In sub-culture 15, a total of nine fragments were detected as polymorphic, which leads to the similarity of 93.7% and variation of 6.2% as shown in Table 2, while in sub-culture number 16, twelve fragments were polymorphic and shows similarity of 86% with a variation of 13.4% as shown in the Table 2.

Table 2. Results of analysis of micropropagated plants of blackcurrant cultivar Ben Sark estimated by RAPD-PCR amplification by using 12 primers.

Sub-culture	Similarity	Variation
Parent	1	0
1	1	0
2	1	0
3	1	0
4	1	0
5	1	0
6	1	0
7	1	0
8	1	0
9	1	0
10	1	0
11	1	0
12	1	0
13	1	0
14	1	0
15	0.94	6.20
16	0.86	13.37

Discussion

The results obtained from the RAPD-PCR analysis of the regenerated micropropagated plants of the blackcurrant cultivar Ben Sark revealed that plants regenerated upto the 14th generation cycle or after 56 weeks in tissue culture environment showed no variation. The evidence appears to suggest that the more recently a cell has been derived from the apical meristem, the less likely it is to display variation. The cells in the tips of the apex are maintained in a continuing embryonic state so that the derivatives have the potential to differentiate into many different tissues within the plant body. It is possible that more efficient DNA repair mechanisms operate in these cells but this would be difficult to demonstrate with present techniques (Hussey, 1986). These results support the rule of SOAEFD Certification of Stocks of soft fruit according to which the maximum time any material may be maintained in culture is one year (52 weeks) or not more than 10 sub-cultures whichever is sooner. After the 14th generation considerable variation was noticed in the 15th sub-culture which increased in the 16th sub-culture. This variation is of considerable importance as it is tissue culture induced and needs to be further explored. It can in no way be confused with epigenetic effects, which may appear by the first *in vitro* cycle (Sibi, 1986).

No report in the literature was found about the use of RAPD-PCR in blackcurrant species for the evaluation of similarity or variation of plants regenerated via micropropagation. Therefore, direct comparison of the RAPDs results obtained here with those of other workers was not possible. However, some workers have reported the existence of somaclonal variation detected using morphological techniques in related soft fruit species such as strawberry. Damiano (1980) reported off-types, these included some

temporary off-type characters (possibly epigenetic) which disappeared after 1-2 years of cultivation while other variation was permanent. One of the first research studies on phenotypic stability in strawberry (Swartz *et al.*, 1981) reported three off-type variants in meristem culture viz., white-streaked leaf chlorosis, an irregular sectorial yellow chlorosis and dwarf-type plants. The dwarf-type plants were characterised by reduced leaf and petiole size, lack runnering, lower yields and smaller size of fruits. Other tissue culture variants included compact trusses as well as runnerless and female-sterile plants in strawberries. Off-type plants regenerated from meristem culture are induced by medium composition, time of culture and number of sub-culture (Shaeffer *et al.*, 1980). These workers have suggested that the frequency of off-type plants increase with the duration of culture. In the present study results generally support this idea.

The dominant nature of RAPDs markers does not allow one to distinguish the dominant homozygote from the heterozygote from the banding patterns. Therefore, for a dominant homozygote at a particular locus, mutations affecting only one of the two alleles would remain undetected. The results presented in this study are indicative of the level of variation and not absolute, because sub-samples were used in this study in terms of selective sub-culture of plants in each sub-culture cycle to reduce the size of the experiment.

RAPDs markers most probably randomly sample the genome. This method appeared simple and the results were reproducible. Because only micro-amount of materials is necessary, this approach can be used to assess each stage of *in vitro* culture. Large sample size can be treated rapidly and the technique lends itself to automation (Williams *et al.*, 1990; Welsh *et al.*, 1991; Hedrick, 1992).

The present study supports the use of RAPD-PCR to give rapid indication of the level of genetic stability of *in vitro* culture regenerated plants. It needs minute amounts of plant material and therefore, it could be used at any stage of micropropagation to get the indicative reflection of the genetic stability of the plants. Ideally to test the genetic integrity of plants regenerated clonal material for a particular species with RAPDs a number of markers equal to or larger than the one required obtaining a saturated linkage map would be desirable. For conifers, this number has been estimated to be larger than 200-300 (Carlson *et al.*, 1991; Neale & Williams, 1991). Once a diagnosis of stability has been obtained with such a number of markers for every targeted species and set of production procedures, genetic stability of *in vitro* propagated plants could proceed with higher confidence on an industrial scale.

Acknowledgement

This research was supported by a grant provided by Ministry of Education, Government of Pakistan.

References

Anonymous. 1981. *Bush Fruits. Reference Book 4.* (Ministry of Agriculture, Fisheries and Food) 1981. H.M.S.O., London.

Carlson, J.E., L.K. Tulsieram, J.C. Glaubitz, V.W.K. LUK, C. Kauffeldt and R. Rutledge. 1991. Segregation of random amplified DNA markers in F_1 progeny of conifers. *Theoretical and Applied Genetics,* 83: 194-200.

Damiano, D. 1980. Strawberry micropropagation. In: *Proceedings of the Conference on Nursery Production of Fruit Plants Through Tissue Culture.* (Ed.): R.H. Zimmerman USDA, pp 11-21.

Doyle, J.J. and J.L. Doyle. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemical Bulletin*, 19:11-15.

Hedrick, P. 1992. Shooting the RAPDs. *Nature*, 335: 679-680.

Hussey, G. 1986. Problems and prospects in the *in vitro* propagation herbaceous plants. In: *Plant Tissue Culture and its Agricultural Applications.* (Eds.): L.A. Withers & P.G. Alderson. Butterworth, pp. 69-85.

Neal, D.B. and G.C. William. 1991. Restriction fragment length polymorphism mapping in conifers and applications to forest genetics and tree improvement. *Canadian Journal of Forest Research*, 21: 545-554.

Nei, M. and W.H. LI. 1979. Mathematical model for studying variation in terms of restriction endonuclease. *Proceedings of National Academy of Science* USA, 76: 569-5273.

Shaeffer, G.W., C. Damiano, D.H. Scott, J.R. McGrew, W.R. Krul and R.H. Zimmerman. 1980. Transcription of a panel discussion on the genetic stability of the tissue culture propagated plants. *Procedings Conference of Nursery Production of Fruit Plant through Tissue Culture-Application and Feasibility.* USDA- Arkansas. pp 64-79.

Sibi, M. 1986. Non-Mendelian heredity. Genetic analysis of variant plants regenerated from *in vitro* culture: epigenetics and epigeneics. In: *CEC Symposium somaclonal variation and crop improvement,* (Ed.): J. Semal. Gembloux, Belgium. pp 53-83.

Swartz, H.J., G.J. Galletta and R.H. Zimmerman. 1981. Field performance and phenotypic stability of tissue culture-propagated strawberries. *Journal of the American Society for Horticultural Science*, 27: 1108-1110.

Welsh, J. and M. McClelland. 1990. Fingerprinting genomes using PCR with arbitrary primers. *Nucleic Acids Research*, 18: 7213-7218.

Williams, J.G.K., A.R. Kubelik, K.J. Hauk, K.J. Rafalkski and S.V. Tingey. 1990. DNA polymorphism amplified by arbitrary primers are useful as genetic markers. *Nucleic Acid Research*, 18: 6531-6535.

(Received for publication 4 November 2000)