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Detection of somaclonal variations in tissue culture-derived date palm plants using isoenzyme analysis and RAPD fingerprints

M.M. SAKER*, S.A. BEKHEET*, H.S. TAHA*, A.S. FAHMY** and H.A. MOURSY*

Plant Cell and Tissue Culture Department and Molecular Biology Department**, National Research Center, 12622 Dokki, Cairo, Egypt*

Abstract

Isoenzyme analysis and activities of peroxidase (PER), polyphenol oxidase (POD) and glutamate oxaloacetate (GOT) and randomly amplified polymorphic DNA (RAPD) fingerprints were used to analyze somaclonal variations in tissue culture-derived date palm plants. The frequency of somaclonal variations was found to be age dependent. Similar isoenzyme patterns for PER and GOT were detected in all analyzed plants. However, variations in activities of the three enzymes studied and in POD isoenzymes were detected. RAPD analysis showed genetic variations in approximately 4 % of the analyzed plants (70 regenerants). The genetic variations were only detected in 6- and 12-months-old cultures. It was observed also that all morphologically abnormal shoots showed genetic variations at the molecular level.

Additional key words: biochemical markers, DNA fingerprints, micropropagation, *Phoenix dactylifera*.

Introduction

The entire tree of date palm is utilized to provide food, shelter, fiber, clothing, furniture and many other by-products. Moreover, date palm tree successfully tolerates extremely adverse environmental conditions, including drought, high temperature and salinity, which are the peculiar criteria of desert lands. Unfortunately, either the improvement of existing palm cultivars or selection of new breeds with superior characters is a tedious endeavour due to the long life cycle of palm tree, its strongly heterozygous nature and impossibility to determine sex before flowering (5 - 7 years). Moreover, palm tree produce very limited numbers of offshoot for transplanting. This situation makes *in vitro* propagation of vital importance.

Although there are many references dealing with date palm tissue culture (*e.g.*, Tisserat 1982, Dass *et al.* 1989, Bhansali *et al.* 1988, Saker *et al.* 1998, Bekheet and Saker 1998), the analysis of tissue culture derived plants for somaclonal variations has yet to be established. Torres (1989), Al-Jibouri and Adham (1990), Baaziz *et al.* (1994), Bannaceur *et al.* (1995) and Booij *et al.* (1995) employed isozyme analysis as a descriptive marker for date palm classification and biodiversity. Recently, RAPD has been introduced in date palm programmes (Shah *et al.* 1994, Fernandez and Tantaoui 1994, Corniquel and Mercier 1994, Saker and Moursy 1998). Very limited data are available regarding molecular analysis of tissue culture derived palm plants. In this context, Salman *et al.* (1988) found in *in vitro* propagated date palms similar isoenzyme patterns and chromosome polyploidization only in one case, suggesting high genetic uniformity of tissue culture derived plants. Recently, a molecular marker linked to somaclonal variations in oil palm has been identified by Rival *et al.* (1998).

Therefore, this study was conducted to employ isozyme and RAPD analysis as simple molecular marker tools for the analysis of somaclonal variations in tissue culture derived date palms.

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Present address of the senior author: Institute for Resistance Research and Pathogen Diagnostic, BAZ, P.O. Box 1505, D-06445 Aschersleben, Germany; fax: (+20) 2 3370931, e-mail: msaker@nrc.sci.eg

Materials and methods

Plants: Tissue culture derived date palm (*Phoenix dactylifera* L. cv. Zaghloul) plantlets were initiated from shoot tip explants according to Bekheet and Saker (1998). This tissue culture protocol has been shown to induce proliferation of embryogenic callus, followed by shoot recovery. Time course needed for the attainment of shoots from shoot tip explants is approximately one year. Cultures used in this study were 3-, 6- and 12-months-old, in addition to one year of establishment. A random sample consisting of 15 - 20 plantlets from each of the different ages were used for molecular analysis. In all cases, young leaves from *ex vitro* plants of the same cultivar were also used and served as control. *Ex vitro* plants were tissue culture-derived plants established for 7 months in permanent soil.

Enzyme activities and isoenzyme analysis: Activities of peroxidase (PER), glutamate oxaloacetate transaminase (GOT) and polyphenol oxidase (POD) were determined according to Chance and Maehly (1955), Reitman and Frankel (1957) and Baaziz *et al.* (1994), respectively.

Electrophoresis was performed for 3 h at constant current 3 mA using mini-vertical electrophoresis unit of *Bio-Rad* (Germany) [7.5 % (m/v) acrylamide slab gels, Tris-glycine buffer, pH 8.3, 0.02 cm^3 of crude extract per well].

Peroxidase isoenzymes were detected on the gel according to Gottlieb (1973a): after electrophoresis, the gel was immersed for 2 - 4 h in the dark at 25 °C in 50 mM sodium acetate buffer, pH 5.5 containing 250 mM hydrogen peroxide and 2 mM guaiacol and was photographed immediately.

Polyphenol oxidase isoenzymes were detected according to Baaziz *et al.* (1994): the gel was immersed in a solution contained 0.1 % (m/v) catechol and 0.1 % (m/v) L-dihydroxyphenyl alanine, solubilized in 0.1 M acetate buffer.

Glutamic oxaloacetic transferase isoenzymes were localized on the gel using Fast blue reaction according to Gottlieb (1973b): the gel was immersed for 2 - 4 h in the dark at 25 °C in 100 mM Tris-HCl buffer, pH 8, containing 30 mM L-aspartate, 15 mM α-ketoglutaric acid and 5 mg of pyrodoxal-5-phosphate. At the end of incubation time, 250 mg fast blue BB salt was added. After visualization for the isoenzyme, the gel was fixed in 10 % acetic acid.

Isolation of genomic DNA and RAPD analysis: DNA was isolated using the CTAB method of Doyle and Doyle (1990). PCR amplification was performed in 0.01 cm^3 reaction mixture containing 20 ng template DNA, 0.5 unit Taq polymerase (*Promega*, USA), 200 µM each of dATP, dCTP, dGTP, dTTP, 10 pmole random primer and appropriate amplification buffer. The mixture was assembled on ice, overlaid with a drop of mineral oil. Amplification was performed for 45 cycles, using UNO thermal cycler of *Biometra* (Germany) as follows: One cycle at 92 °C for 3 min and then 45 cycles at 92 °C for 30 s, 35 °C for 60 s and 72 °C for 2 min (for denaturation, annealing and extension, respectively). Reaction was finally incubated at 72 °C for 10 min and further 10 min at 62 °C. The amplification products were analyzed by electrophoresis in 2 % agarose in TAE buffer, stained with 0.2 µg cm^{-3} ethidium bromide and photographed under UV light.

Results

Enzyme activities: *In vitro* propagation of Egyptian date palm from shoot tip explants, through the proliferation of embryogenic callus, followed by shoot recovery give rise to three types of shoots (Fig. 1*a*). The types can be clearly identified after prolonged periods of subculturing. Normal shoots, comparable to control, were the majority of 3-month-old culture (the actual age of culture is 15 months, because one year is needed for the establishment of shoot culture from shoot tip explants). As a result of prolonged *in vitro* culture, some plants with visible phenotypic variations, including rosy cluster of faint green leaves and twisted leaves can be detected in 6- and 12-month-old cultures. Based on this morphological observations and in order to increase the efficacy of the protocol, it is urgent to develop a simple molecular marker for the identification of genetic variations among tissue culture derived plants. Random samples representing the three types of shoots, in addition to control plant, were analyzed for GOT, PER, POD activities and isozymes. There was no definite relationship between enzyme activities and culture age (Table 1). However, the control plant showed the highest GOT and PER activities, followed by 3-month-old shoots. On the contrary, the highest POD activity was recorded in 12-month-old culture (Table 1).

Isozyme analysis: One GOT isomer with RF 1.6 was detected in the three types of shoots, as well as control plant. Similarly, but with different band intensities, one PER isomer with RF 0.4 was detected. The POD isozymes appeared in two zones: cathodic or slow zone with RF 0.5 and anodic or fast zone with RF 2.5. The cathodic POD was detected in 6- and 12-month-old cultures, while the anodic was detected in control and 3-month-old cultures (Fig. 1*b*).

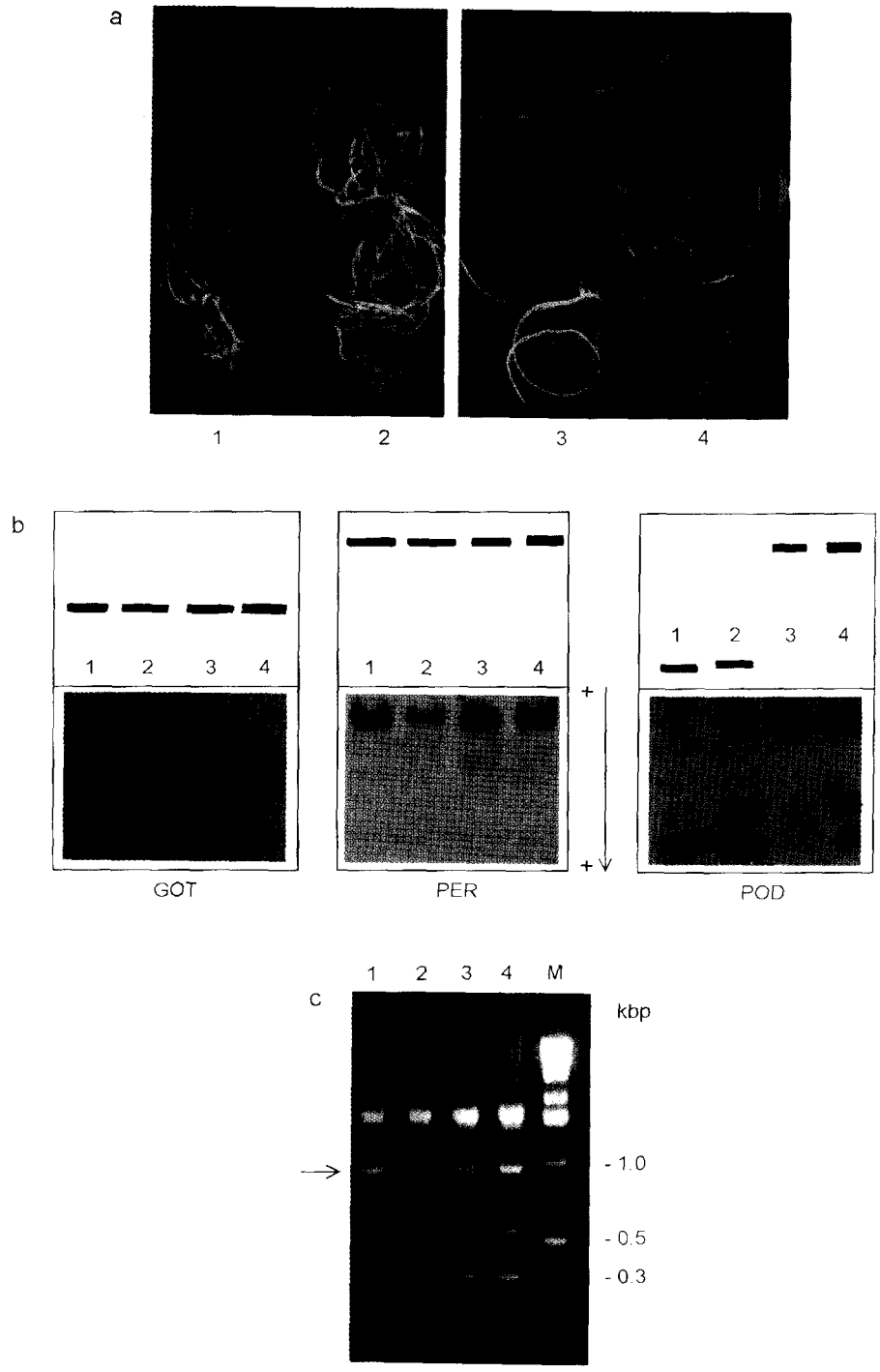

Fig. 1*a*. Examples of date palm plantlets (cv. Zaghloul), representing different ages analyzed for somaclonal variations (*A* - 12-month-old culture, *B* - 6-month-old culture, *C* - 3-month-old culture, *D* - control plant).

Fig. 1*b*. GOT, PER and POD isozyme patterns of date palm plantlets. Letters corresponding to Fig.1. Diagrammatic representations are given above each enzyme.

Fig. 1*c*. Example of RAPD profiles of control date palm plantlets (*lane 1*), 12-, 6- and 3-month-old plants (*lanes 2*, *3* and *4*, respectively), which were generated by PCR amplification of genomic DNA using the random primer (Roth L4). Amplification products were electrophoresed in 2 % agarose using TBE buffer. DNA molecular mass marker of *Gibco* (Germany) is given on the right of the figure and *arrow* at the left of the figure point to the polymorphic band.

Table 1. Effect of *in vitro* culture period (age) on morphology, enzyme activity and isozyme patterns of date palm plantlets (cv. Zaghloul). Means ± SE.

Age	Leaf morphology	Enzyme activity [U μg^{-1}(f.m.)]			RF [cm]		
		GOT	PER	POD	GOT	PER	POD
Control	Normal, straight long and dark green leaves with visual veins	200 ± 4.0	110 ± 2.0	30 ± 0.5	1.6	0.4	0.5
3 months	Normal leaves comparable to the control	190 ± 2.0	90 ± 2.0	35 ± 1.0	1.6	0.4	0.5
6 months	Rosy cluster of faint green, twisted leaves	180 ± 3.0	100 ± 3.0	40 ± 1.0	1.6	0.4	2.5
12 months	Curled faint green leaves	185 ± 1.0	95 ± 0.5	45 ± 0.7	1.6	0.4	2.5

RAPD analysis: DNA isolated from 70 randomly selected shoots representing the different ages, was subjected to RAPD analysis. Based on data from preliminary experiments, thirty random primers giving sufficient amplification products with date palm genome were chosen. Out of the preselected random primers, only three primers gave reproducible polymorphic bands: Roth A4, Roth B8 and Roth L4 (Table 2). PCR amplification using the primer A4 gave one polymorphic band of 500 bp in 6- and 12-month-old cultures only. Meanwhile using the primer B8, two polymorphic bands (300, 400 bp) were detected in control and 3-month-old cultures (Table 2). Using the primer L4, 950 bp band was detected in control, 3-and 6-month-old plants and was not detected in 12-month-old culture (Table 2, Fig. 1*c*).

According to RAPD analysis, 4 % of the analyzed plants (70 regenerants) showed changes in RAPD profiles, which point to genetic variations at the genome level. Genetic variations were recorded only in 6- and 12-month-old cultures (1.5 % and 2.5 %, respectively). No evidences for genetic variations, at the genome or gene expression level (isozymes) were observed in 3-month-old cultures, compared with control plants. It is not worthless to mention that the results of RAPD analysis coincided with that of isoenzyme. However, in few cases, some plants which are classified as abnormal plants based on isoenzyme analysis are not found abnormal with RAPD analysis.

Table 2. PCR amplification with pre-selected random primers gaving polymorphic bands (numbers in parenthesis are molecular masses of polymorphic bands in bp; + - present, – - absent).

Primer	Sequence	Number of ampl. products	Number and size of polymorphic bands	Control	3 month	6 month	12 month
Roth A4	AATCGGGCTG	8	1 (500)	–	–	+	+
Roth B8	GTCCACACGG	10	2 (300, 400)	++	++	– –	– –
Roth L4	GACTGCACAC	9	1 (950)	+	+	+	–

Discussion

The introduction of molecular marker technology in micropropagation programmes of date palm might increase efficiency, because growing of plants in *in vitro* cultures is associated with some genetic modifications termed somaclonal variations (Larkin and Scowcroft 1981). Although, somaclonal variations have been the subject for many studies, few of them deal with somaclonal variations in palms (Rival *et al.* 1998, Salman *et al.* 1988). In the present study, it was found that electrophoretic separations of POD isozymes can give simple and precise method for the screening of somaclonal variations within tissue culture-derived date palm plants. The similarity of POD banding patterns of 3-month-old cultures with their corresponding control plant confirmed the genetic uniformity of plants produced through this micropropagation protocol. Many authors reported the same observations with other crops (*e.g.*, Cecchini *et al.* 1992, Chowdhury and Vasil 1993, Taylor *et al.* 1995).

Genetic variations appeared in 4 % of 6- and 12-month-old plants could be attributed to the extended *in vitro* culture period needed for proliferation of embryogenic callus from shoot tip explants and then shoot regeneration from proliferated callus. The obtained genetic variations may be also induced by 2,4-dichlorophenoxy acetic acid, which was used in the establishment of embryogenic callus from shoot explants. However, the frequency of somaclonal variations in stock

shoot cultures of different ages, detected by POD isozymes and RAPD still acceptable from the practical point of view. Similar findings were reported, *e.g.*, in *Populus* (Rani *et al.* 1995) and wheat (Brown *et al.* 1993). The reasons for somaclonal variations are well stated. Somaclonal variations may be a result of gene amplification, chromosomal irregularities, point mutation and alteration in DNA methylation. Moreover, *in vitro* culture environment may be mutagenic as stated by Larkin and Scowcroft (1981). In conclusion, it could be mentioned that plants which show any type of phenotypic variations are expected to show the same variation at the molecular level. Accordingly, we could suggest that sorting the stock cultures and or decreasing the number of subcultures will probably lead to the reduction of the percentage of plants showing somaclonal variations among tissue culture-derived date palm plants.

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