

The Use of RAPDs Technique for the Detection of Genetic Stability of Date Palm Plantlets Derived From In Vitro Culture of Inflorescence[♦]

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الخلاصة

استخدمت تقانة مؤشرات التضاعف العشوائي المتعدد الأشكال لسلسلة DNA (RAPD) والمعتمدة على التفاعل التضاعفي لسلسلة الدنا (PCR) للتأكد من الثبات الوراثي لنباتات نخيل التمر (*Phoenix dactylifera* L.) صنفى برحي ومكتوم المنتجة من زراعة الأنسجة. استأصلت النورات الزهرية من أشجار بالغة تابعة لصنفي الدراسة واستخرجت منها الشماريخ وقسمت الى قطع صغيرة بطول 0.5 سم وزرعت في وسط MS المحور المجهز بـ 100 مايكرومول من 2,4-D و 15 مايكرومول من 2ip. تم الحصول على نسيج الكالس بعد 8 أسابيع من الزراعة وتكونت البراعم العرضية من هذا الكالس عند نقله الى وسط MS المحور المجهز بـ 10 مايكرومول 2ip و 5 مايكرومول NAA ، جذرت الأفرع الناتجة بنقلها الى وسط MS بنصف قوته التركيبية والمجهز باضافة 5 مايكرومول NAA مع زيادة تركيز السكر في الوسط الى 131.5 مللي مول . تم أقلمة النبيتات الناتجة ونقلت الى التربة بنجاح. اجريت اختبارات البصمة الوراثية باستخدام مؤشرات RAPD إذ استخلص الدنا من عينات الأوراق للأم وعشرة نبيتات نسيجية انتخبت عشوائياً ولصنفي الدراسة . وبعد تحديد الظروف المثلى لتفاعلات RAPD تم الحصول على نتائج واضحة ومتعددة لأنماط RAPD باستخدام 20 بادئ (Primer) انتخبت من أصل 25 بادئ وتم الحصول على

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تطابق تام في نمط توزيع الحزم لسبعة عشر بادئ ولصنفي الدراسة، فيما أظهرت ثلاث بادئات حزماً متباينة لبعض العينات المدروسة بالمقارنة مع بصمة الدنا للنباتات الأم وهي (OPD.01) للصنف برحي و (OPB.07) و (OPC.08) للصنف مكتوم. أوضحت النتائج أعلاه حدوث تغيرات وراثية في النباتات الناتجة من تكوين البراعم العرضية خارج الجسم الحي من أنسجة الكالس المستحث تكوينه من الشماريخ الزهرية لنخيل التمر صنف برحي ومكتوم، وعليه يمكن الأستنتاج ان مؤشرات RAPD هي من مؤشرات الدنا السهلة والسريعة في الكشف المبكر عن التغيرات الوراثية التي قد تحدث في نباتات نخيل التمر الناتجة من زراعة الأنسجة النباتية.

Abstract

The study aimed to use of RAPD – PCR markers to prove the genetic stability of two date palm (*Phoenix dactylifera* L.) cultivars Barhi and Maktom produced by tissue culture technique. Inflorescences were excised from adult tree of the two cultivars. Spadixes were divided in to pieces 0.5 cm in length and cultured on MS modified medium supplemented with 100 µM of 2,4-D and 15 µM of 2ip. Callus was obtained after 8 weeks and adventitious shoots formation was achieved when callus transferred to MS modified medium supplemented with 10 µM of 2ip and 5 µM of NAA. Shoots rooted on half strength MS salts medium supplemented with 5 µM of NAA with increasing sucrose concentration to 131.5 mM. Plantlets were acclimatized and successfully transferred to soil. RAPD – PCR analysis using 25 universal primers were performed on DNA extracted from the fresh healthy leaves of the mother tree and from samples randomly taken plantlets derived from tissue culture. Reproducible RAPD patterns were obtained using 20 primers, Seventeen primers showed completely monomorphic bands in all samples tested of the progeny. Only three primers showed some polymorphic bands on the agarose gel for both cultivars in some samples tested comparing with the DNA banding pattern for the intact trees, these were OPD.01 primer for Barhi, and OPB.07 and OPC.08 for Maktom. According to the results above it was obvious that genetic variations could occurred in plantlets derived from callus proliferated from inflorescence of date palm, furthermore RAPD appears to be an efficient technique and a simple fast DNA marker for the early detection of genetic variations in plants propagated by tissue culture technique.

INTRODUCTION

Date palm *Phoenix dactylifera* L., ($2n=2x=36$) are dioecious, perennial, monocotyledon fruit trees that belong to the family of Arecaceae (1). Dates is the major fruit crop of arid climate region in countries of Middle East and North Africa.

The heterozygosity of date palm makes its progeny strongly heterogeneous (2). Thus the propagation of date palm through offshoots is preferred over the seedlings. Since propagation through offshoots is slow and affected by their low survival rate, tissue culture of female plants has been preferred widely for mass production of true-to-type plants of elite varieties in demand. Propagation by either offshoots or tissue culture generally results in true-to-type plants but some off-types with abnormal phenotypes also develop in tissue cultured plants which may be due to Somaclonal Variations (3).

The use of plant tissue culture techniques through direct or indirect organogenesis by using inflorescences as explants was mentioned by several researchers (4,5,6). Although successful results were reported, no evolution strategies have been used at early stage to assess the uniformity of their tissue-cultured trees. Since long lived plants may have mutants even in apical meristem (7) and during the last few years, variations have been detected among *in vitro* date palm plants (Barhi, Medjool and Khalas) such as Dwarfism, delay in fruiting and fruiting set failure (8, 9, 10,11). All these cases would greatly affect the utilization of tissue culture techniques. Various techniques were used to confirm the true to typeness produced *in vitro*. The morphological markers are based on features like leaf shape, pinnae, fruit stalk and fruit characteristics (12,3). Detection of genetic stability based on morphological markers is difficult. Identification of trees is not usually possible until the onset of fruit, which takes at least five years. Furthermore, a large set of phenotypic data are required that are difficult to assess statistically and are variable due to environmental effects (13). Biochemical markers (isozymes and proteins) have proved to be effective in varietal identification of date palm (14,15). However, they give limited information and are an indirect approach for detecting genomic variation. In recent years and among the different techniques used in generating molecular markers, for direct detection of genomic variation at DNA level, Random Amplified Polymorphic DNA (RAPD) markers have been successfully used for the cultivar analysis and species identification in most plants, due to the technical simplicity and speed of the methodology (16,17,18). The objective of the present study was to employ PCR-RAPD markers for the early detection of genetic variations in date palm inflorescence *in vitro* culture-derived plants.

MATERIALS AND METHODS

Plant material and tissue culture

Floral spathes with immature inflorescences were excised from adult trees of the two cultivars Barhi and Maktom in early spring. Spathes were surface sterilized with 0.1% mercuric chloride (HgCl₂) for 10 min under aseptic conditions, rinsed three times with sterile distilled water and transferred to sterile petri dishes. Spikes were excised and divided into 0.5 cm segments. Explants excised from spathes (8-10) cm with spikes of (3-5) cm in length were cultured on Murashige and Skoog (MS) (19) modified medium supplemented with the following in (mg/l): thiamine-HCl, 1.0; pyridoxine - HCl, 1.0; Nicotinic acid 1.0; Glycine, 2.0; adenine sulfate, 40; myo-inositol, 100; NaH₂PO₄.2H₂O, 170; glutamine, 100; sucrose, 30000; activated charcoal, 2000 and agar-agar 7000 containing 100 µM 2,4-D plus 15.0 µM 2iP for callus induction. The pH of the medium was adjusted to 5.7 with 0.1 N NaOH or HCl, before the addition of agar. The media were dispensed into culture test tubes with 25 ml in each one, and then covered with polypropylene caps. All vials with media were autoclaved under 1.04 kg/cm² of 121 °C for 15 minutes. Adventitious shoots were obtained when callus was transferred to MS liquid agitated medium supplemented with 10.0 µM 2iP and 5.0 µM NAA. Adventitious shoots were elongated, rooted, acclimatized and successfully transplanted in the soil under glasshouse conditions.

DNA extraction

Total cellular DNA was extracted according to the standard procedure (20) with some modifications. About 0.1g fresh and healthy leaves of the intact trees and from ten samples randomly selected plantlets derived from tissue culture, were grounded to a fine powder using liquid nitrogen. Five milliliters of hot (60 °C) 2X CTAB extraction buffer (2% CTAB, 1.4 M NaCl, 0.1 M Tris-HCl pH 8, 20 mM EDTA and 0.2% β-mecaptoethanol) were added, mixed well, and incubated at 60°C in a water bath. After 60 min of incubation at 60 °C with gentle swirling, the resulting cell was lysed and extracted with an equal volume of chloroform/isoamyl alcohol (24:1 v/v). The cell lysate was then centrifuged (4000 g, 20 °C, 15 min). The aqueous phase was transferred into another tube where precipitation occurred by the addition of 0.66 volume of isopropanol. The precipitate was then collected by centrifugation (10000 g, 20 °C, 10 min). Pellets were washed with 70% ethanol, dried and dissolved overnight at 4 °C in 1 ml of TE buffer (10 mM Tris - HCl pH 8.0, 1 mM EDTA). After purification, the resultant DNA was quantified and its integrity was determined after agarose gel electrophoresis as previously described (21)

RAPD analysis

RAPD analysis was carried out by following the described method (22) with few modifications. A total of 25 random decamer primers (Operon Technologies Inc., Alameda, California, USA) were used for RAPD amplification. PCR reactions were carried out in 25 µl volume containing 25 ng of total genomic DNA from each sample for both cultivars, 0.2 µl of a single primer, 100 mM of each dNTPs, 1X PCR buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl, 2 mM MgCl₂) and 1 unit DNA *Taq* polymerase (23). Amplification was performed in a thermocycler (PE 9600) programmed for RAPD: 1 cycle at 94°C for 4 min, and 35 cycles with the following cycle profile: 1 min DNA denaturation step at 94 °C, 2 min annealing step at 36 °C, 2 min extension step at 72 °C and last cycle at 72 °C for 7 min, and an optional soak period at 4 °C. Amplification products were loaded on 1% agarose gel and stained with ethidium bromide (0.5 mg/ml). Amplification for each primer was performed at least twice and only reproducible products were taken. DNA was visualized on a UV transilluminators and photographed using Polaroid black and white film (667-type). Fragments lengths were estimated by comparison with standard size markers (Lambda fage DNA digested with *Hind III* and *Eco R1*).

RESULTS AND DISCUSION

Micropropagation

Callus formation on floral explants was achieved after 12 weeks of incubation in the dark (Fig 1.a). The best response **percentage** to callus formation was 40% and 70% for Barhi and Maktom respectively. Adventitious buds formed on callus occurred after another 12 weeks (Fig 1.b,c,d). Results indicated that 80% of the cultures contain 11.2 and 24.8 buds for the two cultivars respectively was achieved using liquid agitated medium supplemented with 10.0 µM 2ip and 5.0 µM NAA. Shoots produced were rooted after transferred on 5.0 µM NAA and 131.5 mM sucrose added to half-strength MS salts (Fig 1.e). Plantlets were acclimatized and successfully transferred to soil (Fig 1.f).

Genetic Stability of Tissue Culture-Derived Plants

A preliminary experiment was conducted to generate RAPD pattern with 25 primers to identify those that would be suitable in the present study to ensure reproducibility of RAPD marker data, the primers generating no or faint (nonreproducible) bands were discarded (OPA.01, OPB.03, OPC.03, OPD.09 and OPF.05) (Table 1). Twenty primers showed clear and good amplification results. Most of them, about 17, generated monomorphic banding pattern for all samples tested for both

cultivars (Fig.2 a), while three of the twenty primers showed polymorphic banding pattern. These primers were: OPD.01 for Barhi and OPB.07, OPC.08 for Maktom, as shown in Fig. 2(b,c,d). The number of amplified fragments generated by these primers varied from 8 (OPB.7) to 12 (OPD.1) with an average of 10 bands for each sample. Molecular weight scored band range from 0.2 kb to 4900 kb.

Polymorphisms were detected by presence or absence and of molecular weight of amplified fragments for each primer used. As shown in Fig. 2 (b,c,d) that there are genetic changes in the DNA amplification pattern by using OPB.7 and OPC.8 primers for sample 4 of Maktom cultivar when compared to the intact tree and other tested plantlets. DNA amplification pattern for sample 9 of Barhi cultivar showed genetic changes by using OPD.01 primer. Table 2 summarized the genetic variation revealed by RAPD markers.

Table 1. The set of Operon primers used in RAPD reactions

1	OPA.01	6	OPB.03	11	OPC.03	16	OPD.01	21	OPE.01
2	OPA.02	7	OPB.05	12	OPC.08	17	OPD.02	22	OPE.03
3	OPA.03	8	OPB.07	13	OPC.09	18	OPD.05	23	OPE.05
4	OPA.04	9	OPB.09	14	OPC.12	19	OPD.06	24	OPF.01
5	OPA.09	10	OPB.10	15	OPC.19	20	OPD.09	25	OPF.05

: Primer generates monomorphic bands

: Primer generates no bands

: Primer generates polymorphic bands

The findings of this study indicated that genetic variations may exist in *in vitro* culture of date palm inflorescence derived plantlets. Although plant tissue culture has been considered as a mean of vegetative propagation in which a rapid and identical (phonetically and genetically) clones are produced. Somaclonal variations in some plant species may result from changes in nuclear, mitochondrial, chloroplast genome, epigenetic variation as well. It is likely that this is a consequence of tissue culture and could have been induced in the callus production stage due to the use of high concentration of 2,4-D. *In vitro* production of date palm via indirect organogenesis or somatic embryogenesis require the application of relatively high concentration of 2,4-D or NAA for the initiation process (24,25). However these auxin are known to be associated with genetic instability in plants (26,27,28). Callus obtained from tissue culture is highly heterogeneous (29,30) and it is also highly susceptible to mutational change during somatic embryogenesis (31). Frequency of variations depends on some factors: of plant genotype, type of explants,

type of technique used, type and concentrations of growth regulators, age of callus culture or number of subcultures (32,33).

These changes have been explained as the most suitable adaptation mechanism for the new environmental conditions exploiting the plasticity of the plant genome (34). The probable causes include one or more of several processes such as changes in karyotype chromosome number, structure), point mutation, somatic crossing over, sister chromatid exchange, DNA methylation, activation of transposable elements and epigenetic variations (32).

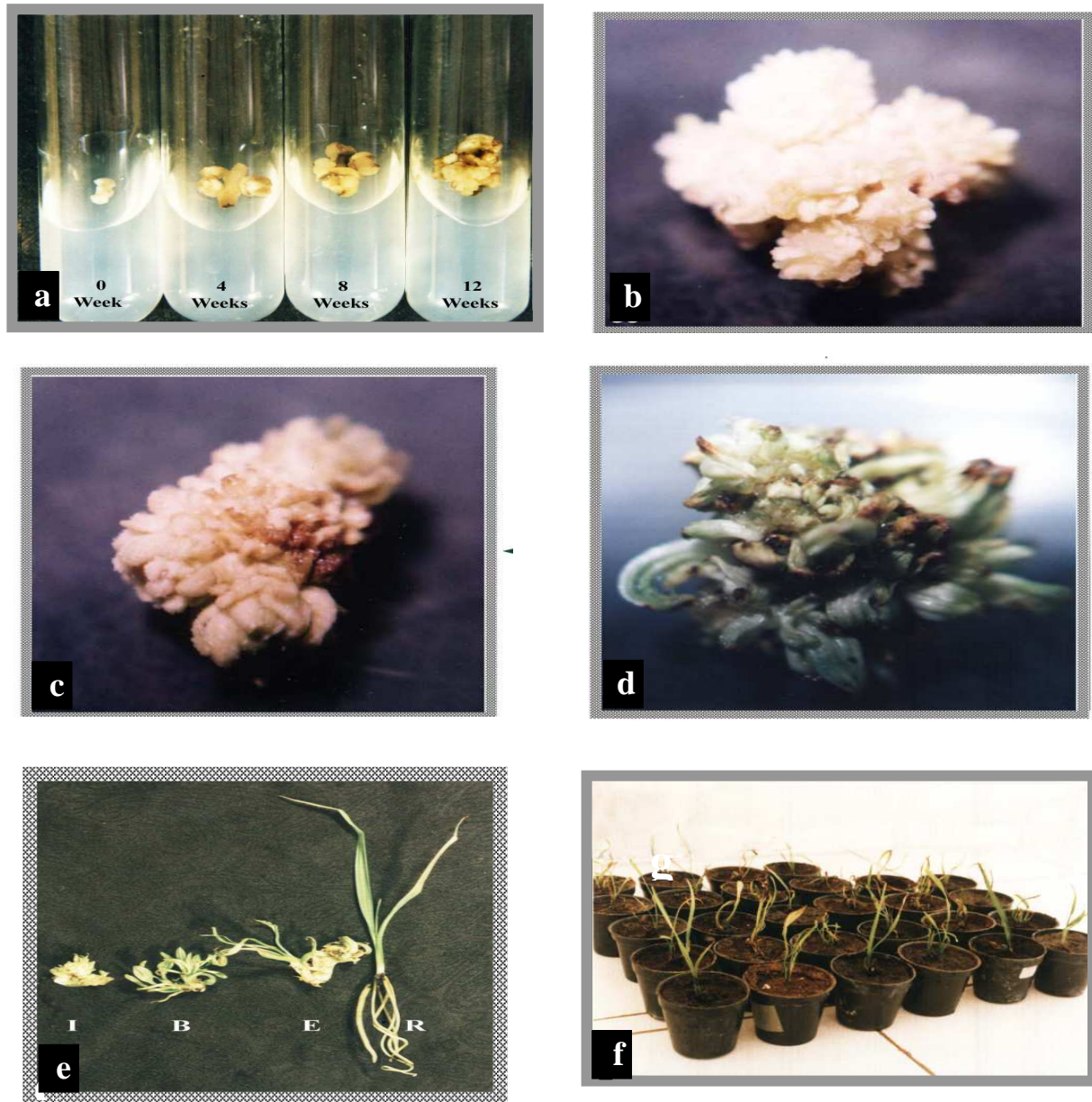


Fig. 1: *In vitro* Micropropagation stages of date palm using inflorescence explants a: Callus formation after 12 weeks b,c,d: adventitious bud formation and development from callus after 0,6,12 weeks respectively e: shoots elongation and rooting f: Plantlets acclimatization in greenhouse.

Table 2. Polymorphisms revealed by RAPD markers using the primers (OPB.07, OPC.08 and OPD.01). (+) presence of the amplified band.(-) absence of the amplified band.

Cultivars	Primers	MW(bp) of polymorphic bands	Intact trees	Samples tested									
				1	2	3	4	5	6	7	8	9	10
Maktom	OPB.07	4300	+	+	+	+	-	+	+	+	+	+	+
		1600	-	-	-	-	+	-	-	-	-	-	-
		800	-	-	-	-	+	-	-	-	-	-	-
	OPC.08	3500	-	-	-	-	+	-	-	-	-	-	-
		1200	-	-	-	-	+	-	-	-	-	-	-
Barhi	OPD.01	1700	-	-	-	-	-	-	-	-	-	+	-

Somaclonal variations can be characterized based on morphological, biochemical (isozymes) and DNA markers such as Randomly Amplified Polymorphic DNA (RAPDs), Restriction Fragment Length Polymorphism (RFLPs), Amplified Fragments Length polymorphism (AFLPs) and others (32). DNA molecular markers have been successfully used to screen date palm plants derived from tissue culture and detect genetic stability (35). RAPD technique has been reported to be useful for studying genetic variation in date palm (36) and the detection of genetic stability of tissue culture derived plants (17,37).

In conclusion, *in vitro* micropropagation of date palm by using inflorescence explants is a promising technique for the mass production of high quality varieties. PCR-RAPD technique is a useful method for the detection of genetic stability of tissue culture derived plants at early stage.

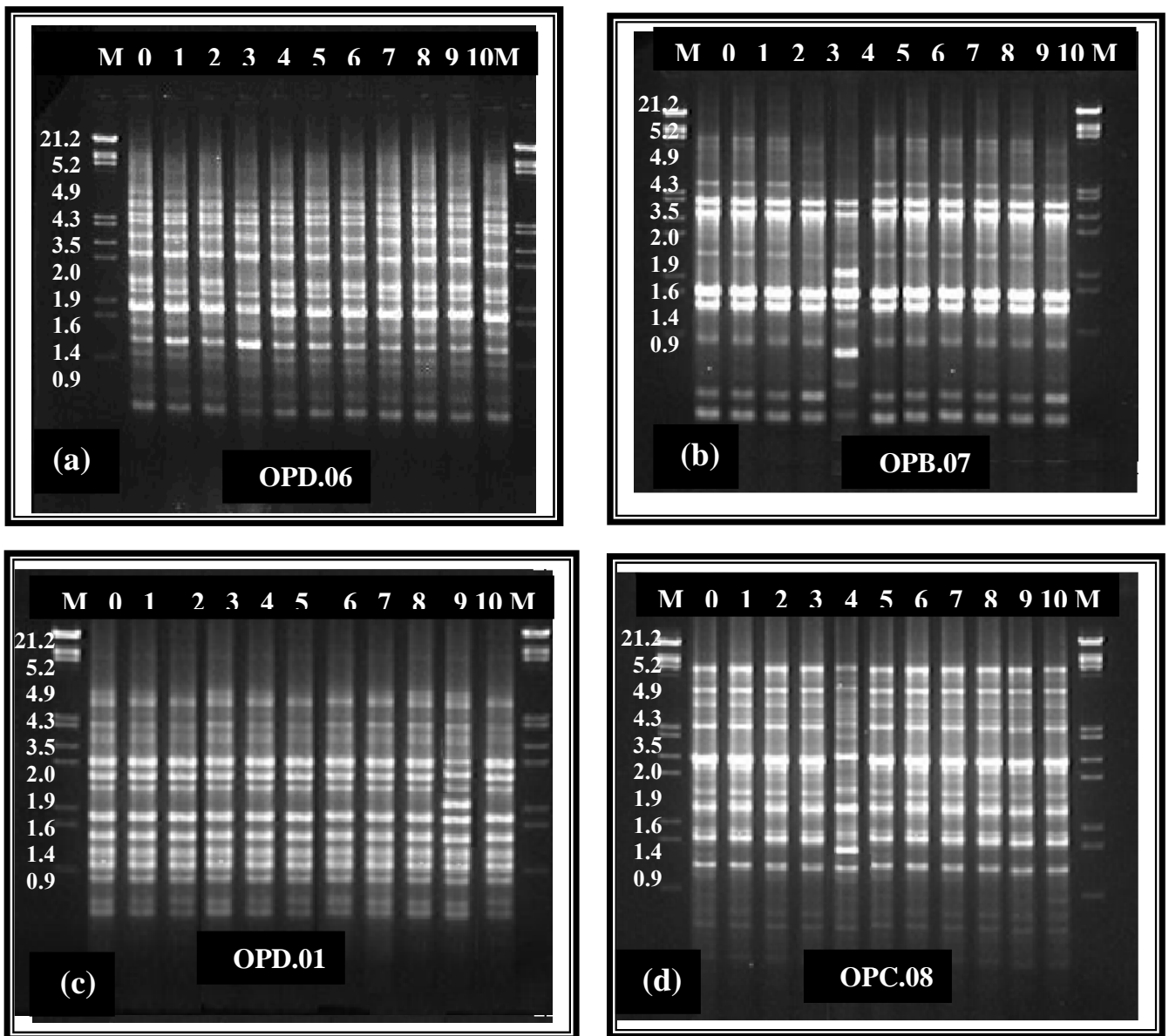


Fig. 2. (a) Monomorphic banding patterns of samples tested revealed by OPD.06 primer for Barhi, (b,c,d) polymorphic banding patterns using the primers (OPB.07, OPC.08 for Maktom and OPD.01 for Barhi), Numbers on the left indicate the fragment size of molecular weight markers (lane M) in kb. The lanes 0 are the banding pattern of the intact trees, while the lanes 1 to 10 are the banding pattern of the samples selected randomly from tissue culture derived plantlets

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