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Somatic embryogenesis in date palm (*Phoenix dactylifera* L.) cultivars and the use of RAPD for detection of genetic stability of regenerated plantlets

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ABSTRACT

The aim of this study was to study the genotype response for *in-vitro* propagation of date palm (*Phoenix dactylifera* L.) and to ensure the genetic stability of plantlets produced by tissue culture *via* somatic embryogenesis through RAPD-PCR technique. To achieve this, three cultivars (Shamran, Halawy and Medjool) were used for callus induction. The nodular creamish-white callus was produced on Murashige and Skoog (1962) medium supplemented with 10 and 25 mg/l 2,4-D + BA (3 mg/l), 2-ip (1 mg/l) and activated charcoal (1.5 g/l). Somatic embryos were produced on MS medium with 0.1 mg/l BA in 30-40 days and they germinated into plantlets on the same medium. In RAPD-PCR of three cultivars, it was found that there was no variation between mother plant and their clones produced *via* somatic embryogenesis.

Key words: Somatic embryogenesis, date palm, callus, genetic stability.

INTRODUCTION

Date palm (*Phoenix dactylifera* L.) is one of the most important horticultural crops cultivated in arid regions. Date palm is usually propagated by offshoots, which are mainly produced during the early life of the palm in limited numbers, depending on variety and other factors. Propagation by seeds is undesirable and impractical due to their dioecious nature. Plant tissue culture techniques have been used to propagate by organogenesis and somatic embryogenesis. Due to the long time requirements for the initiation phase and low multiplication rate, organogenesis in date palm has a low efficiency. Somatic embryogenesis has been achieved by several authors (Zaid and Tisserat, 15; Bhargava *et al*., 4). Although, successful results were reported, no evaluation strategies have been used at an early stage to assess the uniformity of tissue-cultured trees. In the last few years, variations have been noticed in *in-vitro* date palm plants such as delay in fruiting, fruit set failure and dwarf trees (Kaeppler *et al*., 7). These cases greatly affect the utilization of tissue culture for micropropagation. Various techniques are used to certify the trueness-to-type of the plantlets produced and recently developed DNA molecular markers to evaluate tissue culture techniques. Random Amplified Polymorphic DNA (RAPD) markers can be used as a reliable, fast and simple tool to confirm if the palms derived from tissue culture are true-to-type to the mother plant (Ali *et al*., 1; Al-Khalifah and Askari, 3; Othmani *et al*., 11).

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MATERIALS AND METHODS

Offshoots (3-4 year-old and 2-3 kg weight) of date palm cvs. Medjool, Shamran and Halawy separated from adult trees were used as a ex-plant material. The outer leaves were gradually removed till the tender portion was reached. The primordial leaf and apical meristem with sub-apical tissues were excised and kept in anti-oxidant solution (150 mg/l citric acid + 100 mg/l ascorbic acid) for 30 min. without agitation. The explant was surface sterilized with 0.1% $HgCl_2$ containing few drops of Tween-20 with continuous stirring for 10-12 min. followed by washing with autoclaved water for 6-8 times. Shoot tips of 1.5 cm and pieces of internal leaves were cultured on MS solidified medium (Murashige and Skoog, 10) supplemented with additives (100 mg/l inositol, 40 mg/l adenine sulphate, 170 mg/l NaH_2PO_4, 200 mg/l glutamine and 5 mg/l thiamine), 1.5 g/l activated charcoal and following combinations of auxin (2,4-D) (i) 10 mg/l 2,4-D + 3 mg/l BA + 1 mg/l 2-ip, (ii) 50 mg/l 2,4-D + 3 mg/l BA + 1 mg/l 2-ip and, (iii) 100 mg/l 2,4-D + 3 mg/l BA + 1 mg/l 2-ip. The cultures were incubated in dark and 27±2ºC. On the same fresh medium after 3 week interval sub-culture was done, the time taken for callus induction, fresh weight of callus, colour and nature of callus were recorded.

To investigate the effect of supplementation of the culture medium by phyto-hormone on embryogenic callus, embryo formation and germination, creamish-white friable cultures were sub-cultured on the following media (i) MS hormone-free medium, (ii) MS + 0.1 mg/l BA, (iii) MS + 0.5 mg/l BA, and (iv) MS + 1.0 mg/l BA. Embryogenic callus induction per

cent, days taken to maturation of somatic embryos, normal embryos per cent and germination per cent were recorded after five weeks of two sub-cultures. Cultures were incubated under constant light (1000 lux) at 27 ± 2ºC temperature. All culture media were solidified with 0.7% agar and adjusted to pH 5.8 before autoclaving at 1.5 kg/cm^2 for 20 min.

Total cellular DNA was extracted from healthy leaves of offshoots and samples taken randomly from the leaves of plantlets. Around 1.5 g of leaf tissue was ground to a fine powder in liquid nitrogen. The DNA was extracted by Saghai-Maroof *et al.* (12) in modified form. After purification, the resultant DNA (stalked DNA) was quantified and its integrity was determined after agarose gel electrophoresis. A total of 17 random primers (Operon Technologies, USA) were used for RAPD amplification. DNA was visualized on a UV transilluminator and photographed using Polaroid black and white film (667-type). Fragment length was estimated by comparison with standard size markers (λ DNA double digest with *Eco*RI/*Hin*d III).

RESULTS AND DISCUSSION

In this experiment, the effect of auxin (2,4-D) concentrations on callus induction of three cultivars (Medjool, Shamran and Halawy) were investigated. Results presented in Table 1 show that minimum time for callus induction was taken at concentration 10 mg/l 2,4-D + 3 mg/l BA + 1 mg/l 2-ip in cv. Shamran. Also, the fresh weight of callus was found maximum in the same previous medium. Here, it is important to note that as the concentration of auxin increases, the fresh weight and quality (nature and colour) of callus turned darker or become inferior. Cultivar Shamran responded well to callus induction as compared to Medjool and Halawy. From the present results, it is clear that calli were obtained from the shoot tip as well as primordial leaf. This may be due to existance of meristematic tissues. In this respect, shoot tip and primordial leaf explants of date palm has been successfully used for callus induction by several authors (Zaid and Tisserat, 15; Sharma *et al*., 13). Regarding the effect of 2,4-D on callus induction and proliferation, data revealed that addition of 10 mg/l 2,4-D + 3 mg/l BA + 1 mg/l 2-ip enhanced callus induction with reduced time. These results are in line with those obtained by several researchers (Madhuri and Shenkon, 8; Bhargava *et al*., 4; Taha *et al*., 14). They obtained friable and embryogenic callus cultures of date palm by culturing shoot tips and meristematic tissues on medium containing 2,4-D.

The influence of BA (cytokinin) on embryogenic calli induction, somatic embryo maturation, normal embryos and germination into plantlets are presented in Table 2. In this study, the best results of embryogenesis

Table 1. Effect of 2,4-D on callus induction and its quality in datepalm genotypes.

2,4-D (mg/l)	Time taken for callus induction (in days)			Fresh weight of callus (g)			Colour of callus			Nature of callus		
	Shamran	Medjool	Halawy	Shamran	Medjool	Halawy	Shamran	Medjool	Halawy	Shamran	Medjool	Halawy
10	143	163	172	1.39	2.12	2.30	White creamish	White creamish	White creamish	Friable light	Friable light	Friable light
50	154	163	182	0.58	0.88	0.86	Light Brown	Light Brown	Light Brown	Nodulated	Nodulated	Nodulated
100	182	205	210	0.24	0.46	0.38	Brown	Brown	Brown	Compact Nodulated	Compact Nodulated	Compact Nodulated

Table 2. Effect of BA on somatic embryogenesis and their germination in datepalm.

BA (mg/l)	Embryogenic callus induction (%)			Days taken for maturation of somatic embryos			Normal embryos (%)			Germination of somatic embryos (%)		
	Shamran	Medjool	Halawy	Shamran	Medjool	Halawy	Shamran	Medjool	Halawy	Shamran	Medjool	Halawy
0.0	20	30	45	60	65	63	40	25	40	20	30	45
0.1	60	40	80	50	60	45	80	40	70	60	40	80
0.5	50	50	45	45	55	46	60	30	50	50	50	45
1.0	30	15	20	20	40	53	30	10	20	30	15	20

and germination of embryogenic calli were obtained by culturing on 0.1 mg/l BA medium. These embryos were re-cultured on the same medium for germination and subsequent growth and development to plantlets. These results are in accordance with those reported by several authors (Bhargava *et al*., 4; Eshargi *et al*., 5; Taha *et al*., 14).

The study showed that the type of cultivar has a very significant effect on callus and somatic embryo induction. A secific PGR combinations at particular concentration was effective in inducing callus tissue. This study also shows the effect of additives on callus proliferation and somatic embryogenesis. These effects have been reported previously by many researchers (Fki *et al*., 6; Zouine and El-Hadrami, 16; Taha *et al*., 14). Under this study, a pronounced effect of activated charcoal was noticed on minimizing browning of tissue.

On the basis of above observations, primers OPA03, OPA07 and OPA15 were selected to test the somatic embryo generated clones for polymorphism along with mother plants. It was found that there was no variation between mother plant and their corresponding clones based on their DNA banding

Table 3. Screening of RAPD primers for genetic stability analysis in *in vitro* raised date palm genotypes.

Primer	No. of band (s)	Size (bp)	Genotype		
			Shamran	Medjool	Halawy
OPA01	3	1100	+	+	+
		700	+	+	+
		300	+	+	+
OPA03	5	1500	+	+	+
		1400	-	+	+
		1300	+	-	-
		940	+	-	-
		680	+	-	-
OPA04	-	-	-	-	-
OPA05	-	-	-	-	-
OPA06	-	-	-	-	-
OPA07	4	700	+	+	+
		600	+	-	-
		480	+	+	-
		350	+	-	+
OPA08	-	-	-	-	-
OPA09	-	-	-	-	-
OPA10	-	-	-	-	-
OPA11	1	940	+	+	+
OPA12	-	-	-	-	-
OPA13	2	947	+	+	+
		700	+	+	+
OPA14	-	-	-	-	-
OPA15	5	1860	+	-	+
		1150	+	+	+
		730	+	-	+
		600	+	-	-
		530	+	+	+
OPA16	-	-	-	-	-
OPA17	-	-	-	-	-
OPA18	1	600	+	+	+

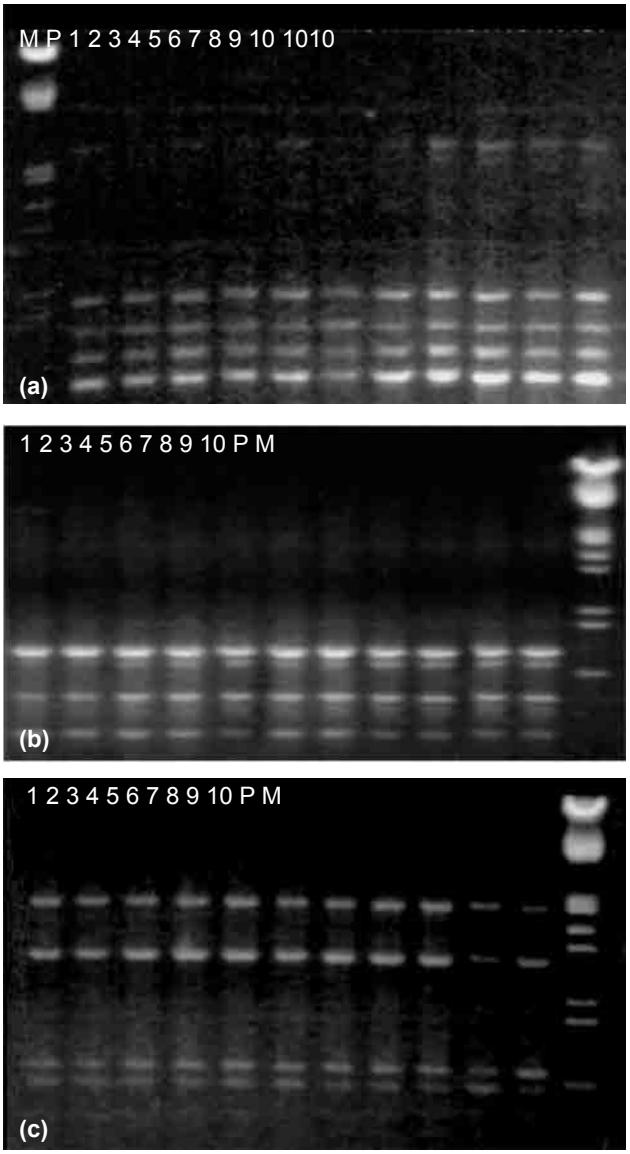

Fig. 2. RAPD profiles generated by OPA-3 with three date palm genotypes Shamran and its clones (a), OPA-7 with date palm genotype Medjool and its clones; (b) OPA-15 with date palm genotype Halawy and its clones; (c) and Lane M- Lamda DNA double digested with *Eco*RI and *Hind* III; lane 1-10- Tissue culture clones (SE), lane P- Parent.

pattern generated by RAPD primer (Fig. 2). Out of 17 OPA series (Table 3) screened, only 3 primers namely OPA03, OPA07 and OPA15 were found to differentiate the genotypes. However, seven primers, *viz.*, OPA01, OPA03, OPA07, OPA11, OPA13, OPA15 and OPA18 could amplify from all three genotypes. Out of these seven primers, OPA03 and OPA15 produced more number of bands (5) followed by OPA07 (4). The amplified bands ranged from 350 to 1860 bp (Table 3). The results of present study were in close conformity to several other authors (Bhargava *et al*., 4; Al-Kaabi *et al*., 2; Othmani *et al*., 11; Moghaieb *et al*., 9). The different stages of *in vitro* multiplication *via* somatic embryogenesis is shown in Fig. 1.

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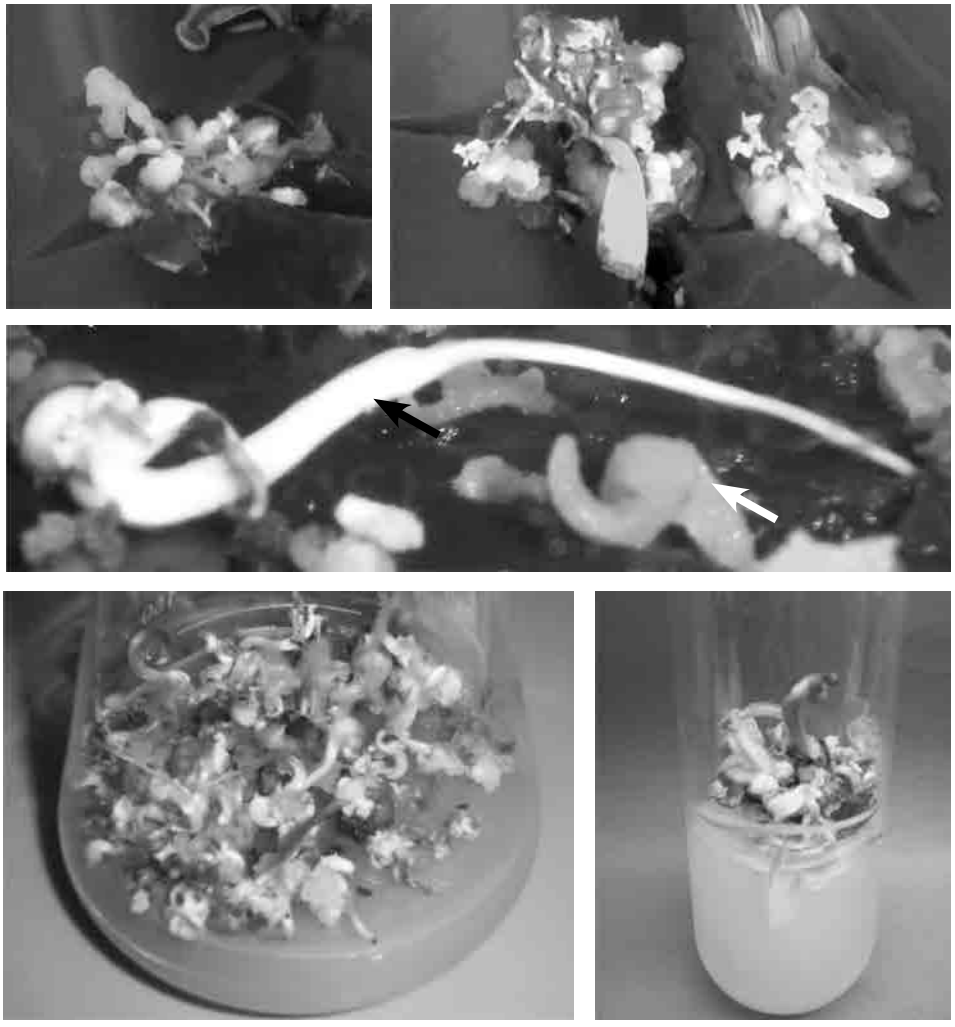

Fig. 1. Influence of 2,4-D and BA on callus induction and somatic embryos. a) Creamish-white callus, b) Brown nodular compact callus, c) Abnormal embryos (black arrow) and normal embryos (white arrow), Influence of BA, d) Somatic embryo germination, and e) shoot proliferation in date palm.

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