Science, Technology and Development 34 (1): 1-8, 2015
ISSN 0254-6418 / DOI: 10.3923/std.2015.1.8
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Evaluation of Genetic Stability in Somatic Embryo Derived Plantlets of Six Date Palm (*Phoenix dactylifera* L.) Cultivars through RAPD Based Molecular Marker

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Abstract: Date palm (*Phoenix dactylifera* L.), is an economically important fruit yielding monocotyledonous and dioecious species. We have established a high regenerative protocol for six important date palm (*Phoenix dactylifera* L.) cultivars (Barhee, Zardai, Khalasah, Muzati, Shishi and Zart) through somatic embryogenesis and regenerated plantlets were successfully transplanted for hardening. But, before using this protocol for date palm forest breeding programs, it is the imperative or desirable to confirm the true-to-type nature of somatic embryos derived plantlets. Here, in the present investigation, Random Amplified Polymorphic DNA (RAPD) technique was used to investigate the patterns and distribution of genetic variability within somatic embryo derived plantlets and their mother plants using seventy primers. Out of the seventy primers, 62 primers produced 558 genetic loci with high quality and reproducibility. The RAPD profile of the somatic embryo derived plantlets showed similar banding patterns to that of the mother plant thus demonstrating the homogeneity of the micropropagated plants. Similarity matrix showed 100% similarity between mother tree and the plantlets derived from somatic embryos by given 1 pair-wise value. This protocol of somatic embryogenesis could thus be successfully used for the mass multiplication and germplasm conservation of date palm forest breeding programme in future.

Key words: Date palm (*Phoenix dactylifera* L.), genetic stability, RAPD, somatic embryogenesis

INTRODUCTION

Date palm (*Phoenix dactylifera* L.), belongs to the family Arecaceae, and is one of the most important cash crops in Middle East as about 90% of the total world production is produced in this region (Zohary and Hopf, 2000; Al-Khayri, 2001; Reilly and Reilly, 2014; Al-Khayri and Ibraheem, 2014). Conventionally, it is propagated sexually through seeds and vegetatively by offshoot (Bonga, 1987; Johnson *et al*., 2013; Shah, 2014). Vegetatively propagated plants accumulate a number of diseases (e.g. bacterial, fungal, viral and mycoplasmal etc.), from air, soil and insect vectors, which decreases their productivity. In addition, the availability of the date palm offshoots is also limited because their number produced by each palm tree is low (Popenoe, 1973) and propagation through seeds has many limitations as well; like low rate of germination and progeny variation, seed dormancy (Venkataramaiah *et al.*, 1980; Chand and Singh, 2004). *In vitro* propagation is a valuable complement to classical breeding strategies (Santos *et al*., 2005; Aslam *et al*., 2012a), which provides an opportunity to develop clones with improved productivity or resistance (Santos *et al*., 2006; Aslam *et al*., 2012b). However, it is already known that tissue culture based technique can induce somaclonal variation like mutation and/or epigenetic changes (Loureiro *et al*., 2007), which may hamper the implementation of clonal forestry programs or, may provide interesting mutants. Somatic Embryogenesis (SE) is the most promising method for clonal mass propagation, because both root and shoot meristems are present (Aslam *et al*., 2012a,b). There have been previous reports on date palm micro propagation through organogenesis and somatic embryogenesis (Al-Khayri, 2001; Aslam and Khan, 2009). In our previous report we have reported *in vitro* high regeneration protocol for six date palm cultivars through somatic embryogenesis (Aslam *et al*., 2011), in the present investigation genetic stability of the somatic embryo derived plantlets was confirmed through seventy Random Amplified Polymorphic DNA RAPD primers.

Random Amplified Polymorphic DNA (RAPD) is one of the most efficient molecular methods in terms of ability to produce abundant polymorphic markers within a short time and limited budget. In this method, a single short primer is used in a Polymerase Chain Reaction (PCR) to amplify random DNA sequences. The RAPDs molecular marker was developed by Welsh and McClelland (1990) and Williams *et al*. (1990). Amplified DNA fragments may be visualized on gel and bands scored as presence/absence character states. Polymerase Chain Reaction (PCR)-based techniques such as Random Amplified Polymorphic DNA (RAPD) and Inter

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Simple Sequence Repeat (ISSR) are immensely useful in establishing the genetic stability of *in vitro*-regenerated plantlets in many crop species (Lakshmanan *et al*., 2007; Joshi and Dhawan, 2007). Random amplified polymorphic DNA do not use radioactive probes as in Restriction Fragment Length Polymorphism (RFLP) (Lakshmanan *et al*., 2007; Jaradat, 2014). Thus, they are suitable for the assessment of the genetic fidelity of *in vitro* and *ex-vitro* raised plantlets (Valladares *et al*., 2006; Ali *et al*., 2009; Celebi *et al*., 2009; Khatri *et al*., 2002; Ganie *et al*., 2012). The aim of this work was to evaluate the genetic stability of the somatic embryo derived date palm cultivars plantlets using RAPD finger printing.

MATERIALS AND METHODS

Somatic embryogenesis and maintenance: Somatic embryogenesis was established according to Aslam *et al*. (2011). Table 1 is showing the detail of date palm cultivars and place of offshoots collections. The pH of all the cultures was adjusted to 5.6-5.8. The media were sterilized in an autoclave for 15 min at 121°C. Cultures were incubated at 25±2°C under a 16 h photoperiod provided by cool pwhite fluorescent lamps (100 M m^{-2} s^{-1} PFD).

DNA isolation: Leaf (1-5 samples) of 6 date palm cultivars (Barhee, Zardai, Khalasah, Muzati, Shishi and Zart) developed through Somatic Embryogenesis (SED) and mother Plant (P) was used as experimental material. DNA isolation was done by the modified method of Doyle and Doyle (1990). Lyophilized leaves (0.5 g) was pulverized in liquid nitrogen. Twenty millilter of CTAB (2% CTAB, 1.4 M NaCl, 20 mM EDTA, 100 mM Tris HCl) buffer was added and incubated at 65°C for 40 min. Equal volume of Chloroform: Isoamyl alcohol (24:1) was added and centrifuged at 6000 rpm for 20 min at room temperature. Aqueous phase was collected in fresh tubes and treated with 10 µL of RNase (50 µg mL^{-1}) at 37°C for 30 min. Equal volume of Chloroform: Isoamyl alcohol (24:1) was added again and centrifuged at 10000 rpm for 20 min at 25°C. About 0.7-1 volume of chilled iso-propanol was mixed to the aqueous phase by inversion and kept at -20°C for one hour. Precipitated DNA was centrifuged at 13000 rpm at room temperature for 20 min. The supernatant was discarded and the DNA pellet was washed with 70% ethyl alcohol. Pellet was then dried, dissolved in sterile water and purified. Following the manufacturer's instructions; the DNA was purified by using HiPurA DNA isolation purification kit.

Table 1: List of six date palm cultivars used and their location

Date palm's cultivar	Place of collection
Barhee	Dubai
Zardai	Abu Dhabi
Khalasah	Fujairah
Muzati	Ras al-Khaimah
Shishi	Sharjah
Zart	Umm al-Quwain

RAPD analysis: The isolated DNA was quantified on 0.8% agarose gel by comparing with uncut Lambda DNA (25 ng μL^{-1}). DNA was diluted to a working concentration of 25 ng μL^{-1}. A master mix comprising of all reaction components except genomic DNA was assembled for multiple reactions as follows. The assay was carried out in 15 µL reaction volume. DNA was amplified with 10-mer random oligonucleotide primers (Table 2). Random amplified polymorphic DNA assay was set up by adding genomic DNA along with the master mix in PCR tubes.

The cycling parameters were: One cycle at 94°C for 4 min, 35 cycles of denaturation at 94°C for 1 min, annealing at 35°C for 1 min, extension at 72°C for

Table 2: List of primers used in RAPD analysis and the number of reproducible bands with size and total number of amplified bands

Primers	Nucleotide sequence (5'-3')	Bands scored	Total number of bands amplified	Annealing temperature	Size of the amplified bands (bp)
OPC1	TTCGAGCCAG	6	80	38	420-810
OPC2	GTGAGGCGTC	5	55	36	680-1300
OPC3	GGGGGTCTTT	11	75	38	450-920
OPC4	CCGCATCTAC	13	83	34	650-980
OPC5	GATGACCGCC	6	43	38	280-700
OPC6	GAACGGACTC	-	-	-	No amplification
OPC7	GTCCCGACGA	9	110	38	760-1200
OPC8	TGGACCGGTG	8	92	32	410-850
OPC9	CTCACCGTCC	10	82	38	250-1200
OPC10	TGTCTGGGTG	6	65	38	650-1700
G1	ATGCTCTGCCC	11	85	34	590-1300
G2	CGGTGACGCAG	12	75	36	400-880
G3	ATGGGGGACTC	5	54	34	550-1700
G4	CGGTCACCTCA	4	54	34	380-1400
G5	AGAGGGCCTTG	10	80	38	500-1950
G6	TGATGACCGCC	5	86	34	750-1750
G7	CAAAGCTGCGG	9	90	32	280-1520
G8	AGTCGCCGTCA	4	86	34	350-1150

Table 2: Continue

Primers	Nucleotide sequence (5'-3')	Bands scored	Total number of bands amplified	Annealing temperature	Size of the amplified bands (bp)
G9	GCCGCGTCTTG	10	90	34	750-1300
G10	CGGACCTGCTG	13	92	38	800-1550
OPN1	CTCACGTTGG	6	95	36	350-1950
OPN2	ACCAGGGGCA	-	-	-	No amplification
OPN3	GGTACTCCCC	9	88	38	550-1550
OPN4	GACCGACCCA	12	90	36	460-1050
OPN5	ACTGAACGCC	10	92	38	400
OPN6	GAGACGCACA	11	83	36	550-1100
OPN7	CAGCCCAGAG	-	-	-	No amplification
OPN8	ACCTCAGCTC	12	32	36	750-2100
OPN9	TGCCGGCTTG	11	45	38	450
OPN10	ACAACTGGGG	6	84	36	550-880
OPS1	CTACTGCGCT	9	80	34	480-900
OPS2	CCTCTGACTG	-	-	-	No amplification
OPS3	CAGAGGTCCC	12	54	32	760-1550
OPS4	CACCCCCTTG	9	46	38	350-870
OPS5	TTTGGGGCCT	10	80	32	400-1400
OPS6	GATACCTCGG	9	82	36	750-1600
OPS7	TCCGATGCTG	8	94	32	890-1500
OPS8	TTCAGGGTGG	13	75	34	450-1080
OPS9	TCCTGGTCCC	10	64	34	410-1800
OPS10	ACCGTTCCAG	3	88	36	280-1500
DPC1	AAGGACAGGG	8	32	34	300-1950
DPC2	AGATTGCACC	7	42	32	950-1750
DPC3	AGGAGCAGAG	12	40	34	550-1080
DPC4	CAACGGAGTG	10	34	36	350-900
DPC5	CACGTGCTAC	-	-	-	No amplification
DPC6	ACGCAGTTCC	11	41	36	660-1400
DPC7	GCGATGGTGG	13	80	36	300-820
DPC8	GTGTCCGGTG	11	74	36	300-1300
DPC9	TCTCTCGCTC	9	56	36	550-1200
DPC10	ATCACGCAGG	6	55	36	690-1300
DPC11	TCGCGCAAAG	-	-	-	No amplification
DPC12	AAACCGCAGG	11	42	38	410-1800
DPC13	CCGATAGGAC	8	34	38	280-1400
DPC14	CTGGTGTCTC	9	74	38	300-1920
DPC15	GGTCACGTAG	5	65	38	930-1550
DPC16	GTTCCCAGAC	10	81	34	480-1720
DPC17	CGAGGAGACC	11	74	34	300-1050
DPC18	GCAGTACGTG	4	63	34	650-1100
DPC19	GCAGTACGAG	9	58	38	700-1650
DPC20	CCGAAGACAC	7	42	32	250-1910
DPC21	AGAGGCTCAC	-	-	-	No amplification
DPC22	GCGTTGACTG	9	32	34	610-1650
DPC23	AGGGCAAACC	8	45	34	560-910
DPC24	CCCGTTACAC	13	42	34	380
DPC25	CATACGCCTC	10	64	32	420-900
OPA1	TCGGCGATAG	-	-	-	No amplification
OPA2	AAGCGGCCTC	13	45	38	560-2100
OPA3	CCAGCAGCTT	10	62	36	350
OPA4	GAGCCCTCCA	9	56	38	280-800
OPA5	GGTCTAGAGG	8	34	32	450-780
Total		558	4116		

2 min, followed by the final extension at 72°C for 10 min and hold at 4°C. The products were size fractioned on 0.8% agarose gel and viewed under UV light.

Data scoring: Scoring of the data for RAPD was performed at 0.8% agarose gels, where clear and distinct amplification products were scored as (1) for presence and (0) for absence of bands. A similarity matrix, based on Jaccard's coefficient has been used to evaluate the pair-wise value between the mother tree and the plantlets derived from somatic embryos. The scoring did not consider differences in intensity of the bands among profiles from different samples.

RESULTS AND DISCUSSION

Embryogenic callus was induced from excised shoot tip on MS medium added with 2, 4-D (1.5 mg L^{-1}). It was maintained by continuous subculturing on fresh nutrient medium and the further rapid development was

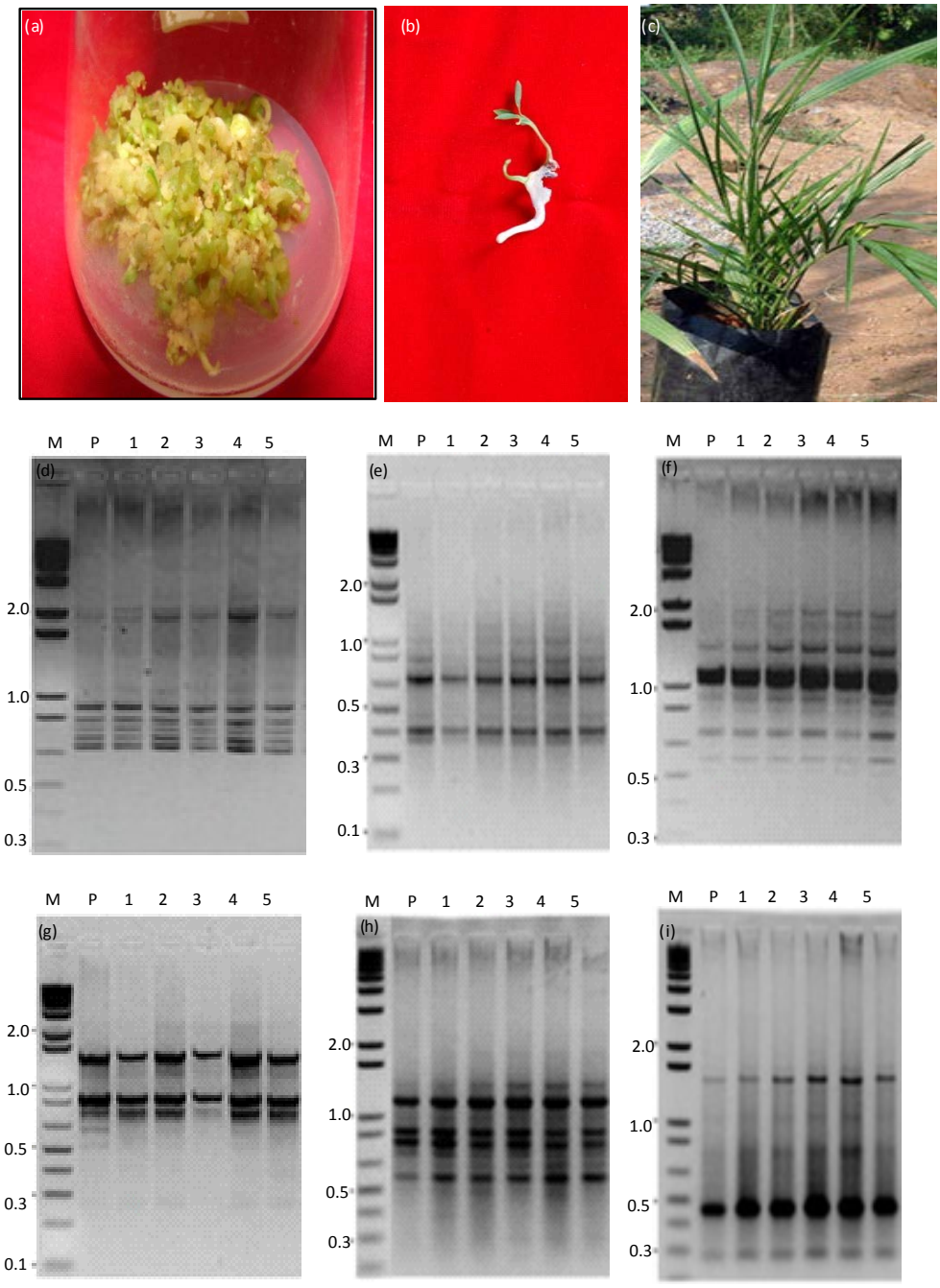

Fig. 1(a-i): Somatic embryos derived date palm cultivars and DNA profiling generated by the RAPD primers OPS 7 in the six different date palm cultivars. M-Molecular marker ((λ DNA restricted with *Hind III* and *Eco RI*), (a) Somatic embryos at maturation stage, (b) Somatic embryo derived plantlet, (c) Successfully growing date palm plantlets in *ex-vitro* conditions, (d) Lane: M (Molecular marker), Lane: P (Mother plant-Barhee), Lane: 1-5 SEDP-Barhee, (e) Lane: M (Molecular marker), Lane: P (Mother plant- Zardai), Lane: 1-5 SEDP-Zardai, (f) Lane: M (Molecular marker), Lane: P (Mother plant- Khalasah), Lane: 1-5 SEDP-Khalasah) (g) Lane: M (Molecular marker), Lane: P (Mother plant-Muzati), Lane: 1-5 SEDP-Muzati), (h) Lane: M (Molecular marker), Lane: P (Mother plant-Shishi), Lane: 1-5 SEDP-Shishi) and (i) Lane: M (Molecular marker), Lane: P (Mother plant- Zart), Lane: 1-5 SEDP-Zart)

Table 3: Similarity matrix for all the generated fragments using RAPD primers

Parameters	Date palm cultivars	Donor tree	Somatic embryos derived plantlets
DT-B	Barhee	1.000	0.954
SESPL-B		-	1.000
DT-Z	Zardai	1.000	0.994
SESPL-Z		-	1.000
DT-K	Khalasah	1.000	0.992
SESPL-K		-	1.000
DT-M	Muzati	1.000	0.996
SESPL-M		-	1.000
DT-S	Shishi	1.000	0.991
SESPL-S		-	1.000
DT-Z1	Zart	1.000	0.992
SESPL-Z1		-	1.000

SEDP: Somatic embryos derived plantlets, DT: Donor tree

achieved on medium containing NAA (1.5 mg L^{-1}) following a suspension culture. Induced somatic embryos were in mixed population and could not be separated out easily. Advanced globular embryos were separated out from the callus mass and placed on MS medium fortified with TDZ (1.0 mg L^{-1}), where somatic embryos started to become greenish morphologically (matured somatic embryos) after 2-3 weeks of inoculation. Somatic embryo germination and plantlet conversion was achieved on MS medium fortified with BAP (0.75 mg L^{-1}). Finally, *ex vitro* transplanted date palm cultivars were tested for genetic fidelity (Fig. 1a-c)

Leaf (5 samples) of somatic embryos derived plantlets of six date palm cultivars (Barhee, Zardai, Khalasah, Muzati, Shishi and Zart) and mother tree (1 sample) was used for RAPD analysis. Seventy 10-mer random oligonucleotide primers were used for the confirmation of genetic stability. Two sets of PCRs were carried out for RAPD finger printing of each sample. Samples of each cultivar were run along with their mother tree with each primer. Only bands reproducible on all runs were considered for analysis. A total of 70 RAPD primers were used for initial screening with the mother plant of concerned date palm cultivars but only 62 RAPD primers gave clear and reproducible bands. Sixty two primers used in this analysis yielded 558 distinct and bands score, 74 for the OPC, 83 for G, 77 for OPN, 83 for OPS, 201 for DPC primers plus 40 fragments for the OPA primers (Table 2), with an average of 9 bands per primer. The entire scored 558 band were monomorphic with in the somatic embryo derived plantlets and their mother plants across all the samples, while there was a polymorphism within the different cultivars. Each primer generated a unique set of amplification products ranging in size from 250 bp (OPC-9) to 2,100 bp (OPN-8 and OPA-2), no polymorphism was detected. All banding profiles from somatic embryo derived date palm cultivars plantlets were monomorphic and similar to those of the mother tree (Fig. 1d-i). A similarity matrix based on Jaccard's coefficient revealed that the pair-wise value between the mother tree and the plantlets derived from somatic embryos was 1, indicating 100% similarity, but it was different amongst the cultivars (Table 3). Table 4 shows the complete distribution of the amplified fragments in six somatic embryo derived date palm cultivars.

Table 4: Distribution pattern of amplified fragments in six somatic embryo derived date palm cultivars

Parameters	Details
Total number of primers screened	70
Total number of primers amplified	62
Total number of bands amplified	4116
Total number of scorable bands	558
Size of the amplified bands	250-2100 bp
Average of scorable band per primer	9

Data pooled from six date palm cultivars originated from somatic embryogenesis and concerned donor tree

Two different types of somatic embryogenesis processes have been observed in plant systems:–Direct embryogenesis (i.e., directly on the explant) and Indirect embryogenesis via an intervening callus phase. In date palm (*Phoenix dactylifera* L.) cultivars, indirect type of somatic embryogenesis has been reported, which include (a) establishment and maintenance of embryogenic tissues from offshoot's shoot tip, (b) proliferation and maturation of embryos and (c) embryo maturation, germination and plantlet conversion. An important characteristic of somatic embryogenesis is its ability of continuous growth in culture (Vengadesan and Pijut, 2009). Both pathways have been reported simultaneously in the some other plants (Dhandapani *et al*., 2008). In date palm somatic embryogenesis has been induced from offshoot shoot tips. The response of a particular type of explants to produce the embryogenic callus varies with explant to explant either in same plant species or different (Gambino *et al*., 2007; Malik, 2008; Aslam *et al*., 2010).

The presence or absence of variations during *in vitro* propagation depends upon the source of explants and the method of regeneration (Goto *et al*., 1998). The sub and supra-optimal levels of plant growth substances, especially synthetic ones, have also been associated with somaclonal variation (Martins *et al*., 2004). Even plants derived from organized meristems are not always

genetically true to the type in many crops (Devarumath *et al*., 2002). Hence, it becomes imperative to regularly check the genetic purity of the tissue culture raised plants in order to produce clonally uniform progeny while using different techniques of micro propagation. The assessment of genetic diversity within and between populations is routinely performed at the molecular level using various laboratory-based techniques such as allozyme or DNA analysis, which measure levels of variation directly. Molecular analyses comprise a large variety of DNA molecular markers, which can be employed for analysis of variation. PCR-based techniques, RAPD were used to test genetic fidelity because of their simplicity and cost-effectiveness. It also amplify different regions of the genome, allows better chances for the identification of genetic variations in the clones (Martins *et al*., 2004). Random amplified Polymorphic DNA has become widely used in various areas of plant research (e.g. population surveys, genetic fidelity of tissue culture raised plants etc.) and it has proved to be a valuable tool in studying inter and intra-specific genetic variations, patterns of gene expression, and identification of specific genes using nearly isogenic variants (Stewart and Excoffier, 1996; Kuddus *et al*., 2002; Dabkevicius *et al*., 2008). Moreover, molecular markers has been used as reliable, powerful and quick tools in the analyses of somaclonal variation in somatic embryogenesis of both conifers and angiosperms (Leal *et al*., 2006; Burg *et al*., 2007; Hornero *et al*., 2001; Wilhelm *et al*., 2005; Valladares *et al*., 2006; Lopes *et al*., 2006; Lopes *et al*., 2009; Munir *et al*., 2011; Pandey *et al*., 2012).

In our study, RAPD analyses did not show any somaclonal variation between the mother tree and somatic embryos derived date palm cultivars plantlets. The total of 558 PCR products analyzed proved a perfect similarity between mother plant and cultivar derived through somatic embryogenesis. Similarly using 32 RAPD primers, Sanchez *et al*. (2003), found no intraclonal or interclonal polymorphism between embryogenic lines originated from the same seedling of *Q. robur*, concluding that these somatic embryos were genetically uniform. Similarly, Valladares *et al*. (2006) also found no evidence of genetic variation within, or between, the embryogenic lines of oak trees. In the specific case of somatic embryogenic lines of *Q. suber*, until the moment, RAPD (Gallego *et al*., 1997), AFLP (Hornero *et al*., 2001) or micro satellites (Lopes *et al*.., 2006) were used to evaluate somaclonal variation among somatic embryos derived plantlets. Studies on somatic embryogenesis genetic stability by Loureiro *et al*. (2005) and of Lopes *et al*. (2006) are in agreement with RAPD analyses to further validate this date palm somatic embryogenesis protocol. The absence of genetic variation using RAPD has also been reported in micro propagated shoots of *Pinus thunbergii* (Goto *et al*., 1998), *in vitro*-regenerated turmeric (Salvi *et al*., 2001), bulblets of Lilium (Varshney *et al*., 2001), *Solanum tuberosum* (Munir *et al*., 2011) and *in vitro* raised sugarcane (Pandey *et al*., 2012).

CONCLUSION

Somatic embryogenesis is a preferred pathway to produce plantlets from *in vitro* raised tissues. In this study, true – to - type nature of the *in vitro* raised clones was confirmed using RAPD -based markers. No differences among somatic embryos derived plantlets and donor tree was obtained. Moreover, plantlets produced through somatic embryogenesis protocol did not show any genetic variability and, the reported protocol may be used to provide true-to-type of date palm cultivars plantlets at large scale.

ACKNOWLEDGMENTS

Board of Trustees of Dubai Pharmacy College are gratefully acknowledged. The authors declare no competing financial interest.

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