Regeneration and analysis of genetic stability of plantlets as revealed

by RAPD and AFLP markers in date palm (Phoenix dactylifera L.) cv. Deglet Nour

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Abstract

The 2,4-Dichlorophenoxyacetic acid induced somatic embryogenesis of Tunisian date palm (Phoenix dactylifera L.) cultivar, Deglet Nour and analysis of the true-to-type conformity of the derived plantlets were investigated in this study. For this purpose, two polymerase chain reaction (PCR)-based methods namely, randomly amplified polymorphic DNA (RAPD) and amplified fragment length polymorphism (AFLP) markers were used. Data proved that the modified Murashige and Skoog (MS) media including 1, 10 and 100 mg.l-1 2,4-Dichlorophenoxyacetic acid have permitted an intensive callogenesis when leaves are incubated in dark. Subcultures on MS medium supplemented with 0.1 mg.l-1 2,4- Dichlorophenoxyacetic acid stimulated a rapid maturation of somatic embryos in light. A mean of 120 somatic embryos were developed from 0.5 mg callus within 3 months. Embryos germination and conversion to plantlets were successfully achieved after transfer to free plant growth regulators MS medium. On the whole, 75% progenies survival was established in soil.

In addition, RAPD and AFLP analyses were performed in 180 randomly selected plantlets. The resultant DNA banding profiles exhibited similarities between the mother plant and its progeny. This result strongly supported the true-to-type nature of the in vitro derived progenies in date palms.

Keywords Amplified fragment length polymorphism (AFLP), Date palm, plantlets, Random Amplified Polymorphic DNA (RAPD), plant regeneration, somatic embryogenesis

Abbreviations: 2,4-D, 2,4-Dichlorophenoxyacetic acid; IBA, Indole-3-Benzylaminopurin

Introduction

The date palm (Phoenix dactylifera L.), (2n=36), an out-breeding heterozygous dioecious perennial monocot is characterized by large genetic diversity. This fruit crop is of great importance in the oasis development not only for date production but also for the maintenance of socio-economical and environmental stability of the arid areas. Due to its out-breeding and heterozygous nature, date palm progenies consisted of 50 % of male trees and 50 % of females that are not true-to-type (Carpenter and Ream 1976). Therefore, its conventional propagation is made by offshoots. However, this method is very limited in time and in number to establish new date palm plantations. Moreover, several genotypes did not produce offshoots and those issued from other cultivars are difficult to root. In addition, seed-propagation palms do not bear true to type and required up to seven years before fruiting stage. In order to overcome these hybridization difficulties, in vitro multiplication methods have provided alternative strategy either for mass propagation of elite cultivars or for date palm improvement. For instance, somatic embryogenesis is reported to be a relatively consistent strategy for genetically homogeneous plant micropropagation (Kanita and Kothari 2002). It should be stressed that since in vitro culture may cause disturbances in the genome organization of regenerated plantlets inducing somaclonal variation (Larkin and Scowcroft 1981) conformity of the derived plants constitutes the main criteria for large scale use particularly for new groves establishment. Therefore, certification of the derived plants' conformity is required. For this purpose, different methods have been reported and described the use of the Williams et al. (1990) random amplified polymorphic DNA (RAPD) and the Vos et al. (1995) amplified fragment length polymorphism (AFLP) methods have been reported as reliable, quick and inexpensive procedures to identify clones and cultivars and to assess somaclonal variation (Taylor et al. 1995; Trifi et al. 2000). The present study portrays the achievement of the in vitro propagation of the Tunisian date palm elite cultivar, Deglet Nour, through somatic embryogenesis and the assessment of derived progenies' certification using RAPD and AFLP markers.

Materials and methods

Plant material

Juvenile leaves of 1-3 cm in length sampled from 20 years old date palm cv. Deglet Nour were used. These were randomly collected from trees growing in plantations at El Mahassen located in the south of Tunisia. 180 somatic embryo-derived plantlets produced in different media as well as the mother tree were used to carry out the designed analyses.

Tissue culture Media and culture conditions

Leaf sections of 1 cm2 were sterilized by soaking in 0,01 % $HgCl_2$ for 1 hour, three times washed in sterile distilled water and cultured on different MS media (Murashige and Skoog 1962) containing 5 % of sucrose (w/v) and 0.7 % of Difco agar (w/v). As reported in Table 1, 0.0, 1.0, 10, and 100 mg.l-1 of 2,4-D were added to M1, M2, M3, and M4 media respectively.

The pH was adjusted to 5.7 prior to autoclaving at 1.4 Kg cm-2 for 20 min. Production of callus from explants was accomplished via incubation of cultures in the dark at 28 ± 2 °C and regular subculture at an interval of 6-7 weeks for 4-5 months under the same culture conditions. Experiments consisted of at least 25 cultures per treatment and were repeated three times.

For testing differentiation of embryogenic callus, the entire expanding explants with resultant embryogenic callus were transferred to MS medium supplemented 1 mg.l-1 2,4-D. Cultures were placed in air-conditioned culture room at 28 ± 2 °C with 16/8 h photoperiod providing 80 µmol m-2 s-1 fluorescent light and subcultured every 1 month.

To regenerate plantlets, matured somatic embryos were picked from maturation medium after 2 months of culture and transferred to free plant growth regulators MS medium without any postmaturation treatment. Transfer of plantlets to free-living conditions was made as follows: plantlets are carefully removed from agar medium avoiding the root system damage and washed in distilled water for 15 min to remove excess adhering media and to avoid their dehydration. Plantlets were than rinsed three times using distilled water, sprayed with 0. 5% benomyl fungicide solution and transferred to soil.

DNA extraction

Total cellular DNA was isolated from the young leaves according to Dellaporta et al. (1983). Quantification and integrity of the resultant DNAs was spectrophotometrically performed using a GeneQuant spectrometer (Amersham, Pharmacia, France) and analytic agarose gel electrophoresis according to Sambrook et al. (1989), respectively.

Primers and RAPD assays

Nine universal primers purchased from Operon (Alameda, USA) identified as OPA04, OPA07, OPA16, OPC07, OPD05, OPD06, OPD16, OPD19 and OPE16 were used to perform RAPD amplifications (Table 2). These oligonucleotides have been reported to generate reproducible amplification and revealing inter varietal polymorphisms within date-palms (Ben Abdallah et al. 2000).

Amplifications were conducted in a total volume of 25 µl including: 60 ng of total cellular DNA (~1 µl), 150 µM of dNTP (dATP, dGTP, dCTP and dTTP), 3 mM of $MgCl_2$, 30 pM of primer, 2.5 µl of Taq DNA polymerase buffer (10×), 1 U of Taq DNA polymerase (Amersham, France). Mixtures were firstly heated at 94°C during 5 min as a preliminary denaturation step before entering 45 PCR cycles including each one: 30 seconds at 94°C for the denaturation, 1 min at 37°C for primers' hybridization and 2 min at 72°C for complementary strands synthesis.

A final elongation during 10 min is usually programmed at the end of the last amplification cycle. PCR products are electrophoresed by loading 12 µl of each reaction in 1.5 % agarose gel using TBE (1×) buffer during 2 h, stained with ethidium bromide and visualized under UV transilluminator (Sambrook et al. 1989).

Primers and AFLP assays

Primers used in this study and AFLP assays were performed as reported in Rhouma et al. (2007). Six set primers were tested in this study. These are identified as follows: EAAC/MCAA, EAGC/MCAA, EAAC/MCAG, EACA/MCAG, EACC/MCTA, EAAC/MCAT. ENNN/MNNN where E and M correspond to the EcoR1 and Mse1 restriction enzymes respectively.

The AFLP banding patterns were electrophoresed on denaturing polyacrylamide gels (6%) and visualised after silver staining according to Chalhoub et al. (1997).

Results and discussion

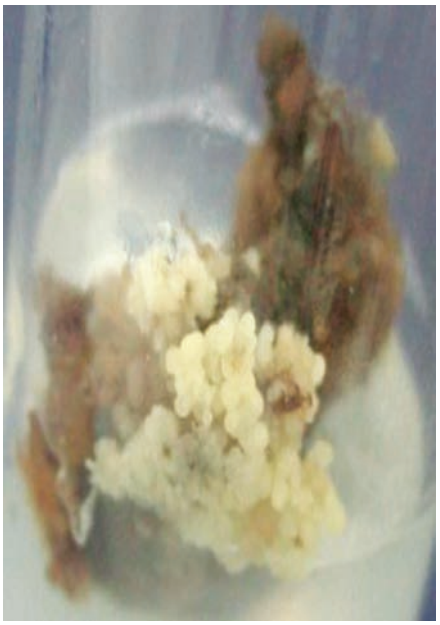
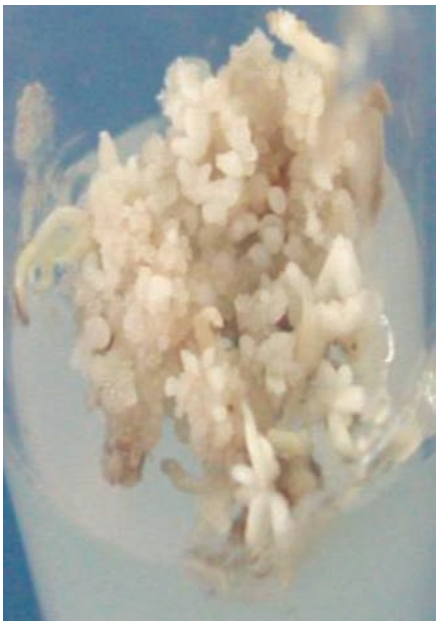
Callus production, differentiation and plant regeneration

Depending on the concentration of the 2,4-D, different morphogenetic responses were scored from leaf explants cultured during five months. Note worthy that explants of 1cm2 size exhibited irregular growth and died after 8 weeks of culture when cultivated on MS medium deprived of 2,4-D. Juvenile leaf pieces have initiated, however, friable calli, showing miniature (< 2 mm) white nodules when cultivated on the medium M2 (Fig. 1a). In fact, these calli appeared either on the upper or the lower surface of the explants. Similar results have been reported in date palms (Drira and Benbadis 1985). Our data therefore concur with the efficiency of the 2,4-D as a callus-inducing agent in this crop. In addition, direct embryogenesis was induced on this medium (i.e. M2) at the basic part leaves without any callus development (Fig. 1b). Therefore, we assume that the 2,4-D is very efficient to promote callus growth without any somatic embryos on the callus either on similar media or on those used for subculture. However, decrease of 2,4-D concentration till to 0.1 mg.l-1, has permitted to induce embryogenic calli. These have proliferated normally and yielded an average of 45 elongated somatic embryos per 0.5 g fresh weight of embryogenic callus (Fig. 1c). As illustrated in figures 1c and 1d, maturation and germination of the derived embryos have been successfully achieved when the 2,4-D is completely removed from the culture medium. Such results have been reported somatic embryos of other plants as Momordica charantia (Thiruvengadam et al 2006), and oil palm (Aberlenc-Bertossi et al 1999).

Juvenile leaf explants were also able to produce embryogenic callus more efficiently on M3 medium than on M2 medium. Indeed, the frequency of callus induction on M3 medium was 45% on average vs. 30% on M2 medium. It should be stressed that explants cultivated on the MS medium supplemented with 100 mg.l-1 2,4-D (M4) turned regularly to brown and most of them died. However, addition of activated charcoal to the nutrient medium has permitted to neutralize these phenomena and to enhance the explants' endurance and their ability to embryogenic callus with a frequency of 20%. This is in agreement with properties of this adsorbent in the decrease of the toxic browning of explants and the plant embryogenesis process as reported by Touchet (1991) and Sharma et al. (1980).

These were developed into matured stage (Fig. 1d) and germinated when the 2,4-D was completely removed from the culture medium. These results confirm those from by Thiruvengadam et al. (2006), with somatic embryos of Momordica charantia L and by Aberlenc-Bertossi et al. (1999), with somatic embryos of oil palm.

The derived plantlets (Fig. 1e) were hardened through growing in ½ MS liquid medium supplemented with 1mg.l-1 IBA. Prior high intensity illumination incubation in this medium was necessary before their transfer to a soil mixture (Fig. 1f). Finally, regenerated plants were transferred to non-sterile conditions for acclimatization and to conditions of gradual decrease of humidity levels. According to these conditions, 80 % of plantlets have easily subsisted during one month and their transfer to soil field conditions was successfully achieved (Fig. 1G).


Plantlets stability as revealed by molecular markers

In order to examine the genome stability of the derived plantlets, we have designed two PCRDNA based methods: the random amplified polymorphic DNA and the amplified fragment length polymorphism. Starting from a set of 180 plantlets, reproducible monomorphic RAPD banding patterns have been obtained using all the tested primers. Figure 2 illustrated typical examples of DNA profiles produced using the OPE16 (panel a) and OPD05 (panel b).

All the primers screened, were found to amplify a total of approximately 60 bands. The number of bands for each primer varied from 1 to 9, with an average of 5 bands per primer (Table 2). These results indicate the efficiency of the RAPD technique to highlight the diversity of the plantlet DNA; Further, whatever the used primer, the RAPD banding patterns were constant within both all plantlets and the plant mother which unambiguously showed the absence of variation about both the number and the position of the obtained amplified DNA fragments for each one of the tested primer.

Thus we assume that strong genome stability characterizes the in vitro progenies according to the designed experimental conditions. In addition, as reported in figure 3, similar results have been registered in the AFLP banding patterns. In fact the six primer pairs tested for their ability to generate AFLP banding patterns from DNA corresponding to the plant mother together with all the derived in vitro plantlets yielded a total of 200 bands ranged in size from 100 - 600 bp with a mean of 58.33 fragments per primer combination. Figure 3 illutrates typical examples of AFLP banding profiles generated by EACA/MCTA primers' combination.

Analysis of the ALP banding patterns exhibited no variation about the number and the size of AFLP bands either among the progeny profiles or between progeny and the mother plant one. This result strongly supported the genome stability reported above since the used primers combination have been reported as consistent tools to evidence polymorphisms in this crop (Rhouma et al. 2007). Therefore, taking into account the derived banding patterns via RAPD and AFLP analyses, we assume that a genome conformity is observed in the resultant plantlets suggesting that the 2,4-D didn't induce somaclonal variation in date palms. Such result is for great importance for the scaling-up of the designed process aiming at mass clonal micropropagation of date palm. Similar results have been reported in plantlets regenerated from embryogenic suspension cultures in the Tunisian date palm Deglet Nour cv. (Fki et al. 2003). In fact these authors have described the use of flow cytophotometry analysis to examine the ploidy level of the plantlets studied and revealed identical ploidy level in the mother plant and its in vitro progeny. Moreover, among 100 microsatellite alleles, difference about only one allele size has been registered in one plantlet over 150 studied (Zehdi et al. 2004). Similar results have been reported in other crops through

Fig 1:

somatic embryogenesis such as in (Tautorus et al. 1991; Michaux-Ferrière et al. 1992; Heinze and Schmidt 1995; Vasil 1995; Cohen et al. 2004).

One possible explanation to this fact would be related to conservative forms of generating the embryogenic lines, namely, embryo cleavage, as is the case in conifers (Tautorus et al. 1991) or multicellular budding (Michaux-Ferrière et al. 1992). Besides, somatic embryogenesis is claimed to be less prone to genetic alterations because it entails the expression of many different genes (Vasil 1995).

Nevertheless, genetic variation in somatic embryogenesis has been reported in other crops (Rotino et al. 1991; Hawbaker et al. 1993; Ostry et al. 1994; Isabel et al. 1995). On that subject, it is obviously necessary to enlarge both the number of plantlets and/or the number of primers to best cover the genome. Even so, it will be interesting to try other techniques used to detect genomic diversity such as the Random Amplified Microsatellite Polymorphism (RAMPO) and the Single Strand Conformational Polymorphism (SSCP). At the moment, research is currently in progress in order to clear up this problem.

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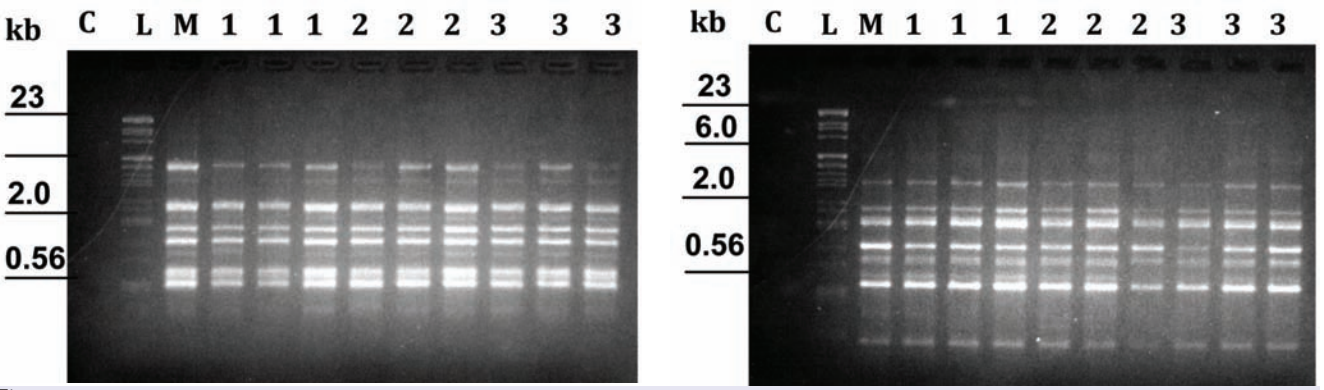

Fig 2

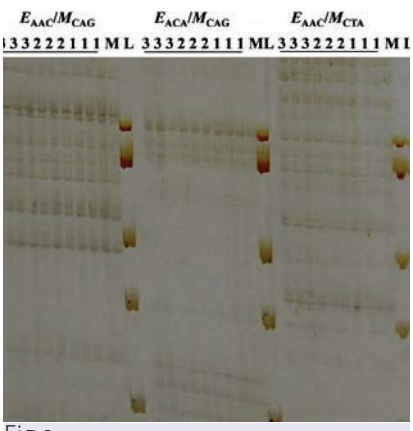

Fig 2

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Table 1: Composition of media used for date palm in vitro regeneration

Meduim composition (mg l-1)	M1	M2	M3	M4
MS salts	4,568	4,568	4,568	4,568
MS vitamins	1	1	1	1
Fe-EDTA	65	65	65	65
Sucrose	50,000	50,000	50,000	50,000
Myo-inositol	100	100	100	100
Glycine	2	2	2	2
Glutamine	100	100	100	100
KH_2PO4	120	120	120	120
Adenine	30	30	30	30
Difco agar	7,000	7,000	7,000	7,000
2,4-D	0	1	10	100
Activated charcoal	300	300	300	300

Table 2: Type of primers used and the number of generated bands.

primer	Sequence	Bands number
OPA-04	AATCGGGCTG	5
OPA-07	GAAACGGGTG	7
OPA-16	AGCCAGCGAA	6
OPC-07	GTCCCGACGA	9
OPD-05	TGAGCGGACA	7
OPA-06	ACCTGAACGG	5
OPA-16	AGGGCGTAAG	6
OPA-19	CTGGGGACTT	9
OPE-16	GGTGACTGTT	6
Total		60

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Fig 1: Induction of somatic embryogenesis and plant regeneration from leaf explants of date palm cv. Deglet Nour. (a) Embryogenic callus within proembryogenic globular structures obtained after 6-month culture period on MS medium including 1 mg.l-1 2,4-D (M2). (b) Direct embryogenesis at the basic part of a juvenile leaf cultured on MS medium including 1 mg.l-1 2,4-D for 6 months of culture. (c) Initiation of differentiation of embryogenic callus after 1 month of transfer on MS medium supplemented with 0.1 mg.l-1 2,4-D. (d) Matured somatic embryos obtained after 10 weeks of transfer of differentiated embryogenic callus on MS medium deprived of 2,4-D. (e) Hardened-plantlets with full radicle and shoot obtained after 3 months of transfer to ½ MS liquid medium supplemented with 1mg.l-1 IBA. (f) Potted
plantlets 3 month after transfer to a green house. (f) two years plants old after transfer to free-living conditions. (g). Scale bar: (a)10 mm; (b) 5 mm; (c)10 mm; (d) 20 mm; (e) 15 mm; (f) 100 mm; (g) 300 mm

Fig 2: RAPD DNA banding profiles generated using OPE16 primer (panel a) and OPD05 primer (panel b). Negative control (C); Molecular size marker (L); Mother plant (M); plantlets from media including 1, 10, 100 mg.l-1 2,4-D respectively (lanes 1, 2, 3)

Fig 3: Typical examples of AFLP banding profiles generated by EACA/ MCTA primers' combination. Standard molecular size marker (L); Mother plant (M); plants from media including 1, 10, 100 mg.l-1 2,4-D (lanes 1, 2, 3 respectively). ENNN/MNNN, primer pairs, E and M for EcoR1 and Mse1 restriction enzymes respectively