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Full Length Research Paper

Regeneration and molecular analysis of date palm (*Phœnix dactylifera* L.) plantlets using RAPD markers

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Clonal plants of date palm (*Pœnix dactylifera* L.) were regenerated from juvenile leaves on regimes consisting of the use of 2,4-D. Success depended on the concentrations of 2,4-D tested. The cultures produced adventitious shoot buds directly at the basal part of leaves as well as excessive calli. Somaclonal variation in plantlets which can be induced by 2,4-D during recurrent somatic embryogenesis were tested by random amplified polymorphic DNA (RAPD) profiles. Nine arbitrary 10-mer primers were used to amplify DNA from 180 plantlets. RAPD patterns of the plantlets were identical with the original plant mother, indicating that 2,4-D did not induce somaclonal variation that can be detected by the RAPD technique.

Key words: Date palm, 2,4-dichlorophenoxyacetic acid, random amplified polymorphic DNA, somaclonal variation, somatic embryogenesis.

INTRODUCTION

The date palm, *Pœnix dactylifera* L., is one of the most economically important fruit tree in the desert areas of the Middle East and in North-Africa. Its cultivation is established with selected ecotypes clonally propagated via offshoots. However, this method is relatively slow, since a limited number of offshoots are produced by a date palm tree during its lifetime.

Seed-propagation of date palm does not bear true to type fruits due to heterozygosity and it requires several years to reach the adult fruiting stage. Extensive efforts are being made to mass propagate date palm through *in vitro* tissue culture (Drira, 1983). However, plantlets derived from calli, in other species, have sometimes exhibited genetic variations different from the parental clone (Larkin and Scowcroft, 1981), particularly when the sub-culturing period is prolonged, because of the non-uniform nature of callus tissue (Kanita and Kothari, 2002). Hence, plantlets produced through tissue culture via callus should be suspected as being potentially aberrant. Further, work is needed to verify the clonal nature of date palm plantlets.

Among molecular markers, random amplified polymerphic DNA (RAPD) analysis (Williams et al., 1990) has been used as a reliable, quick and inexpensive method to identify clones and cultivars (Trifi et al., 2000) and to assess somaclonal variation (Taylor et al., 1995).

This paper describes a current progress involved to regenerate plantlets through *in vitro* tissue culture from juvenile leaves explants of an elite date palm (*Phoenix dactylifera* L.) cultivar, Deglet Nour, the most important commercial cultivar in Tunisia, accounting for 70% of the national production, and studying the genetic stability of

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Abbreviation: AIB: Indole-3-butyric acid; **BAP**: 6-Benzylaminopurine; **NAA**: α-Naphthalenacetic acid; **2,4-D**: 2,4-Dichlorophenoxyacetic acid; **MS**: Murashige and Skoog medium.

Table 1. Media composition used for date palm *in vitro* regeneration.

Medium composition (mg l^{-1})	M1	M2	M3	M4	M5	M6	M7
MS salts	4,568	4,568	4,568	4,568	4,568	4,568	4,568
MS vitamins	1	1	1	1	1	1	1
Fe-EDTA	65	65	65	65	65	65	65
Sucrose	50 000	50 000	50 000	50 000	50 000	50 000	50 000
Myo-inositol	100	100	100	100	100	100	100
Glycine	2	2	2	2	2	2	2
Glutamine	100	100	100	100	100	100	100
KH_2PO_4	120	120	120	120	120	120	120
Adenine	30	30	30	30	30	30	30
Difco agar	7 000	7 000	7 000	7 000	7 000	7 000	7 000
2,4-D	0	1	10	100	0	0	0
NAA	0	0	0	0	1	0.1	0
BAP	0	0	0	0	1	0	0.1
Activated charcoal	300	300	300	300	0	0	0

its plantlets by RAPD patterns.

MATERIALS AND METHODS

Plant material

Juvenile leaves obtained from an adult date palm tree (*Phoenix dactylifera* L. cultivar Deglet Nour) growing in open fields in Elmahassen, Tunisia. Molecular analyses were done from young leaves obtained from both 180 plantlets and original mother plant.

Media and culture conditions

Leaf sections of 1 cm^2 were surface sterilized by soaking in 0.01% H_gCl_2 for 1 h, and rinsed three times in sterile distilled water and cultured on media M_1, M_2, M_3 and M_4 (Table 1). Cultures were maintained in the dark at 28 ± 2°C and subcultured at an interval of 6 weeks for 4 - 5 months under the same culture conditions. Experiments were repeated at least three times and at least 25 cultures were employed per treatment. Production of calli from explants was performed in the dark. For testing shoot organogenesis and obtaining morphogenetic responses from calli, cultures were exposed to 16 h/8 h, light/dark photoperiod at a diurnal temperature of 28 ± 2°C and a nocturnal temperature of 22 ± 2°C. The subcultures were done after every month.

Chopping and division of calli

500 mg of callus was chopped by grinding through a 500 µm mesh filter. The small pieces obtained were recultured aseptically on a fresh solid medium of the same composition but devoid of hormones.

Rooting and acclimatization

To induce roots, shoots were excised and transferred into a full-strength MS medium (Murashige and Skoog, 1962) supplemented with 1 mg/l AIB. The rooted plantlets were washed with tap water 15 mn to avoid dehydration and removing excess adhering media. Before their transfer to the soil medium, young plants were sprayed with 0.5% benomyl fungicide solution.

DNA extraction

DNA from young leaves of adult tree and from 180 plantlets was extracted following the method of Dellaporta et al. (1983). After purification, DNA concentration was determined and its integrity was proved after agarose minigel electrophoresis according to Sambrook et al. (1989).

DNA amplification

Nine universal primers (Operon, Alameda, USA) that gave a reproducible amplification which revealed an-inter varietal polymerphism with date palm (Ben Abdallah et al., 2000) were tested. They are OPA04, OPA07, OPA16, OPC07, OPD05, OPD06, OPD16, OPD19 and OPE16. The reactions were conducted in a total volume of 25 µl that contains: 60 ng total cellular DNA (~ 1 µl), 150 µM of dNTP (dATP, dGTP, dCTP and dTTP), 3 mM of $MgCl_2$ and 30 pM of primer. The reactional mixture was preliminary denatured for 5 min at 94°C before adding 1 U of Taq DNA polymerase. Afterwards the tubes were placed in a thermocyclar which programmed to carry out 45 cycles of 30 s at 94°C for the denaturation, 1 min at 37°C for primer 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 cycle amplification. The amplification products were separated by electrophoresis in 1.5% agarose gel. After migration during 2 h under a constant intensity of 45 mA, The DNA was visualized on gels under the UV with presence of ethidium bromide (Sambrook et al., 1989).

RESULTS AND DISCUSSION

Initiation of callus and shoots

Following five months in culture, differential morphogenetic responses were obtained from leaf explants depending on 2,4-D concentration (Table 2). No benefit resulted from culturing excised juvenile leaves on nutrient media devoid of 2,4-D. In fact, such explants exhibited erratic growth. However, leaf pieces, when cultured on MS medium supplemented with 1 mg/l 2,4-D (M2) initia-

Table 2. Morphogenetic responses of leaf explants of date palm grown on M1, M2, M3 and M4 media after 5 months of culture.

Medium	Percentage of callus production	Percentage of shoot formation
M_1	0	0
M_2	15	10
M_3	40	2
M_4	2,5	0

Figure 1. Embryogenic callus originating from a juvenile leaf of date palm on medium M2. Magnification: 2x.

ted friable calli, showing minute (< 2 mm) white nodules (Figure 1). These calli appeared on either the upper or the lower surface of the leaves and also on the base part of leaves where they are attached to the rachis; the latter was clearly the more competent, giving rise to the larger part of the calli obtained.

These results agree with those from previous studies where 2,4-D was used as callus-inducing agent in date palm (Masmoudi, 1999). Furthermore, adventitious shoot buds were produced directly on the same medium at the basal party of leaves, without the intervening formation of calli (Figure 2). This callus induction and direct shoot regeneration in response to *in vitro* culturing on media with 2,4-D also has been observed with date palm (Drira and Benbadis, 1985).

Culturing explants on MS medium containing 10 mg/l 2,4-D (M3) was the most interesting in term of enhancing callus production. In contrast, this medium gave consistently poor shoot formation as compared to M2. When explants cultured on MS medium supplemented with 100 mg/l 2,4-D (M4), leaf explants usually turned brown and most of them died; adding of activated charcoal to the nutrient medium was necessary to counteract the phenomena for the initial survival of the explants and its subsequent proliferation. These results corroborate other investigators who indicate the importance of this adsorbent in both plant embryogenesis process (Touchet 1991) and the minimisation of toxic browning (Sharma et al., 1980).

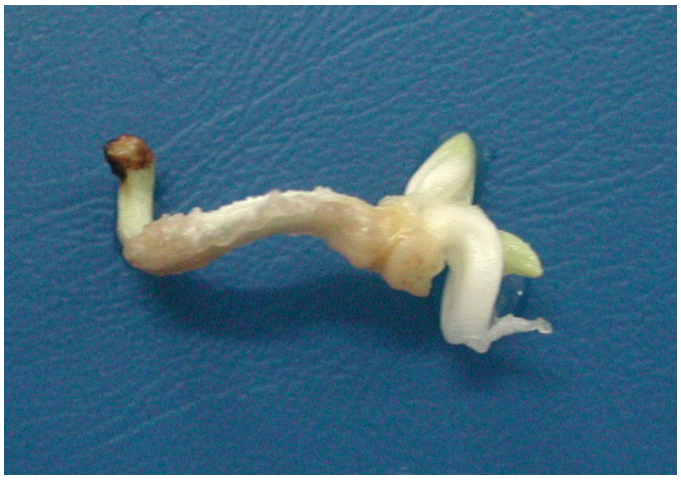

Figure 2. Shoots development originating from the basal party of a juvenile leaf on medium M2. Magnification: 2x.

From this study it emerges that the date palm multiplication via *in vitro* culture using leaf pieces of trees offers tremendous potential for an inexpensive method for its large-scale propagation without destroying the elite material. Among the 2,4-D doses tested, the dose of 10 mg/l, was superior to the other concentrations in accelerating callus induction, however best shoot development occurred on media containing 1 mg/l. But in all cases, propagation of the date palm cultivar Deglet Nour can be realized under both light (1 mg/l) and high (100 mg/l) doses of 2,4-D.

Regarding to the precise origin of callusing and shoot formation, it has been noted that basal part of leaves were more responsive than its surface to direct and indirect plant regeneration.

Callus differentiation

Differentiation of calli into embryogenic nodules was initiated by transferring 4 week old calli to hormone-free medium containing activated charcoal (M1) and incubating it under light (Figure 3). Otherwise, we have noted that in certain cases, the culture of young leaves in presence of 1 mg/l 2,4-D ended in embryos formation via direct embryogenesis from the basal party of leaves (Figure 4) without a callus phase.

Our results showed that chopping of callus into small pieces accelerated its differentiation (Figures 5 and 6) and resulted in a high increase in its fresh weight per 6 weeks (Figure 7). Thus, chopping of callus into small

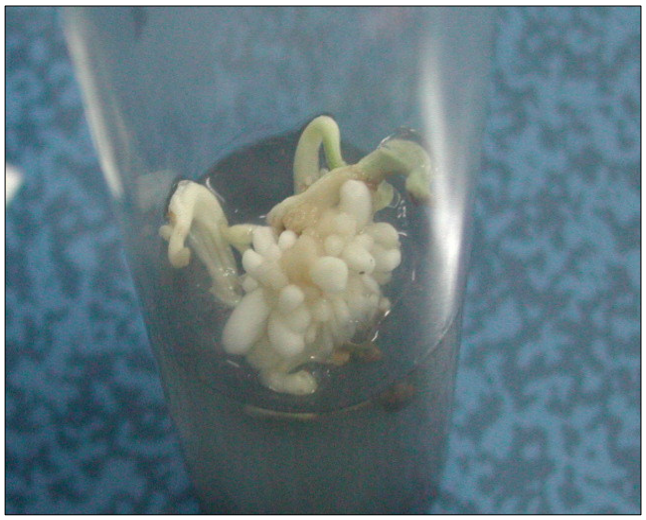

Figure 3. Differentiation of callus into embryonic nodules on medium M1. Magnification: 2x.

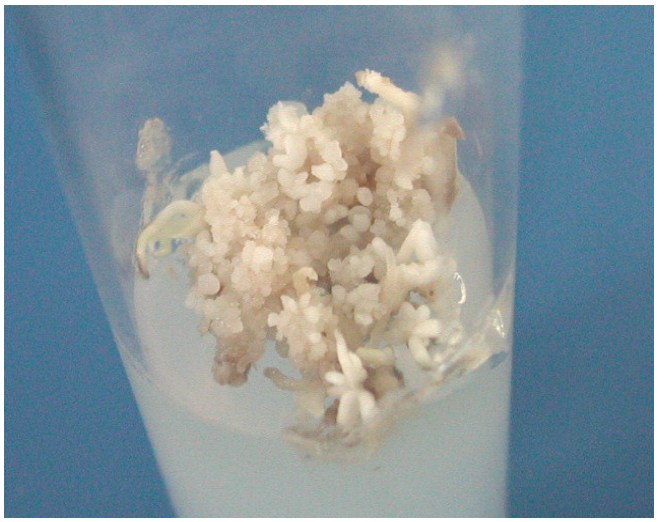

Figure 5. Proliferation of 4-week-old non-chopped callus after 40days of culture on medium M1. Magnification: 2x.

Figure 4. Young embryos formation via direct embryoge-nesis from the basal party of a leaf on medium M2. Magnification: 2x.

Figure 6. Proliferation of 4-week-old chopped callus after 40days of culture on mediumM1. Magnification: 2x.

pieces is one of the best approaches for date palm micropropagation. The reason for this is unknown but could be related to an inhibitory substance(s) released by surrounding embryos. Hence, chopping of callus might raise this inhibition; consequently, embryos enter into lengthening phase.

Shoot proliferation

When shoots were cultured on MS medium supplemented with NAA and BAP each at 1 mg/l (M5) and devoid of activated charcoal, their proliferation was very intensive (Figure 8). In contrast, the culture of shoots on free-hormone MS medium (M1) decreases enormous-ly its proliferation ability (Table 3).

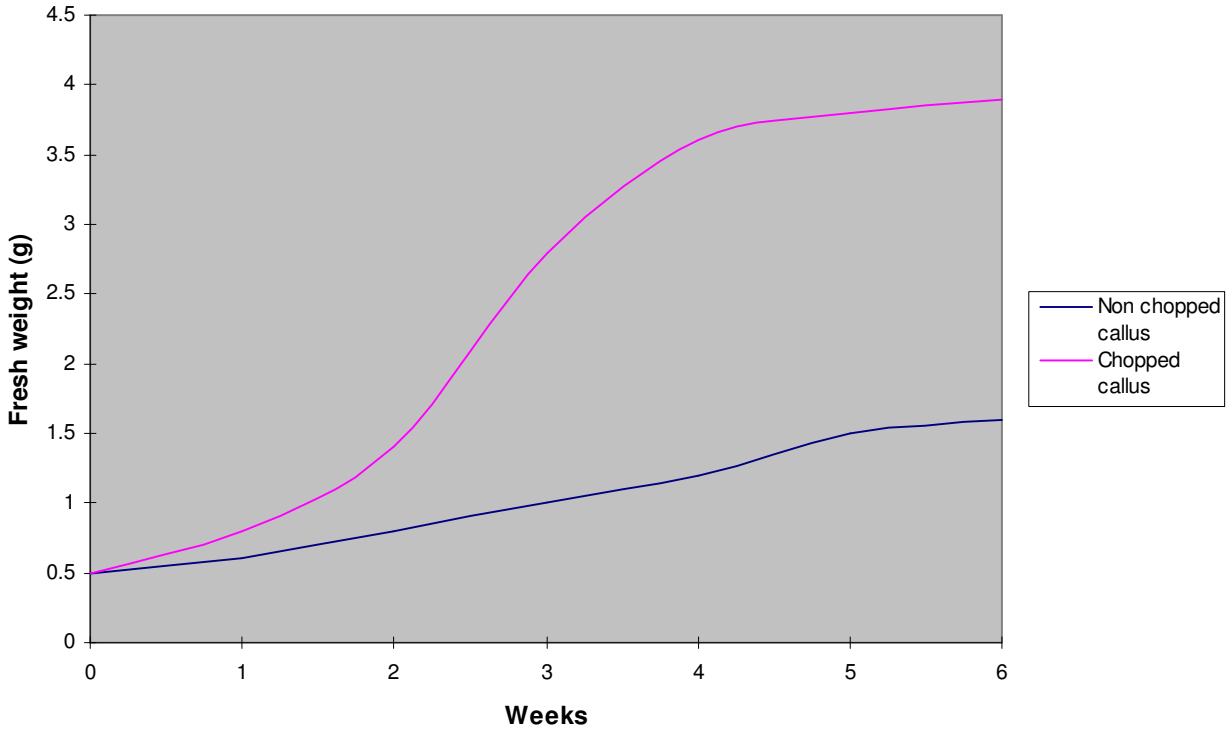

Figure 7. Changes in biomass during 6 weeks of chopped and non chopped date palm callus on M1 medium.

Table 3. Average number of shoots induced per explant of date palm in media M1 and M5 after 1 month of subculturing.

Medium	M1	M5
Number of shoots per explant	11	32

Table 4. Percentage rooting obtained from shoots of date palm in media M1, M6 and M7.

Medium	M1	M6	M7
Percentage rooting	15	85	30

Rooting and acclimatization

In the present study, elongated shoots were rooted successfully on a full-strength MS medium containing 0.1 mg/l NAA but without BAP (M6). Interestingly, rooting capability deteriorated in the presence of 0.1 mg/l BAP (M7). A pronounced decrease in root formation was observed on an MS medium that lacked growth regulators (Table 4).

Plantlets were hardened through growing in liquid medium containing half the strength of MS, supplemented with 1 mg/l AIB, coupled with incubation under high intensity illumination prior to transfer to the soil mix in small pots.

Figure 8. Shoots proliferation on medium M5. Magnification: 2x.

Eventually, regenerated plants were transferred to non-sterile conditions for acclimatization and to conditions of

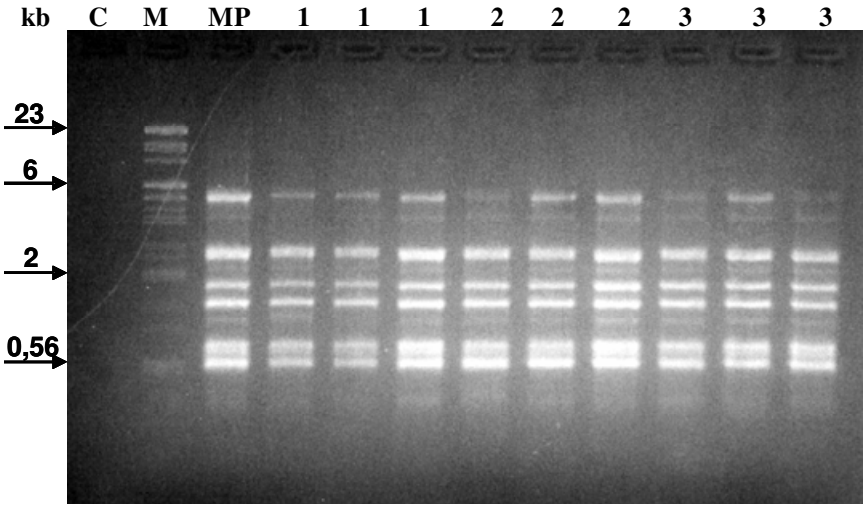

Figure 9. DNA profiles generated by primers OPE16. C, control; M, size marker indicated in kb; MP, sample of original plant mother genotype; 1, samples of genotype obtained in media M2; 2, samples of genotypes obtained in media M3; 3, samples of genotypes obtained in media M4.

steadily decreasing levels of humidity. After one month, 80% of regenerants had survived. The regeneration diagram that we have adopted is characterized by the succession of three phases. The first one includes the establishment of organic-embryogenesis clusters which takes place in the darkness and remains the more complex stage determinates in this process. The differentiation, regeneration of embryos and shoot rooting phases that coming after depends on both of the nature, the dose and the period of hormonal treatments earlier applied.

Analysis of plantlets diversity

All the nine primers screened, were found to give reproducible patterns. Typical examples of amplification profiles were illustrated in Figures 9 and 10. These primers amplified 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 5). 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 original plant mother which clearly showed the absence of variations that concern both the number and the position of the obtained amplimers for each one of the tested primer. Hence, these results are in favour for the hypothesis of conformity of the obtained plantlets in our experimental conditions, suggesting that 2,4-D did not induce somaclonal variation, a result which opens the way for the further scaling-up of this process for mass clonal micropropagation of date palm.

Table 5. Type of primers used and 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
OPD-06	ACCTGAACGG	5
OPD-16	AGGGCGTAAG	6
OPD-19	CTGGGGACTT	9
OPE-16	GGTGACTGTT	6
Total		60

Similar results were obtained with softwood plantlets regenerated through somatic embryogenesis (Heinze and Schmidt 1995). Furthermore, Flow cytometry analysis has revealed that plantlets regenerated from embryogenic suspension cultures and original plant mother of date palm cultivar Deglet Nour showed the same ploidy level (Fki et al., 2003). 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, there are several reports on genetic variation in somatic embryogenesis (Rotino et al., 1991; Hawbaker et al., 1993; Ostry et al., 1994; Isabel et al., 1995). On that subject, it is obviously necessary to en-

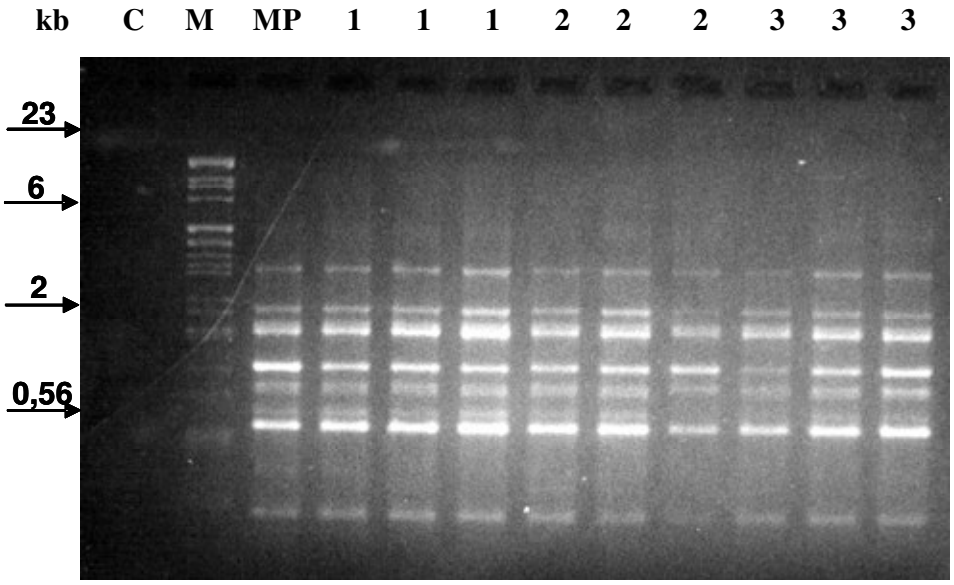

Figure 10. DNA profiles generated by primer OPD05. C, control; M, size marker indicated in kb; MP, sample of original plant mother genotype; 1, samples of genotype obtained in media M2; 2, samples of genotypes obtained in media M3; 3, samples of genotypes obtained in media M4.

large 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), the Single Strand Conformational Polymorphism (SSCP) and the Amplified Fragment Length Polymorphism (AFLP). At the moment, research is currently in progress in order to clear up this problem.

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REFERENCES

Ben Abdallah A, Stiti K, Lepoivre P, Dujardin P (2000). Identification de cultivars de palmier dattier (*Phœnix dactylifera* L.) par l'amplification aléatoire d'ADN (RAPD) Cahier d'études et de recherche Francophones/Agricultures. 9(2) : 103-107.

Dellaporta SL, Wood J, Hicks JB (1983). A plant DNA minipreparation. Version II.Plant Mol. Biol Rep., 1: 19-21.

Drira N (1983) Multiplication végétative du palmier dattier (*Phoenix dactylifera* L.) par la culture *in vitro* de bourgeons axillaires et de feuilles qui en dérivent. C R Acad. Sci. Paris. 296: 1077-1082.

Drira N, Benbadis A (1985). Multiplication végétative du palmier dattier (*Phoenix dactylifera* L.) par réversion, en culture *in vitro* d'ébauches florales de pieds femelles adultes. J. Plant Physiol. 119: 227-612.

Fki L, Masmoudi R, Drira N, Rival A (2003). An optimised protocol for plant regeneration from embryogenic suspension cultures of date palm, *Phoenix dactylifera* L., cv. Deglet Nour. Plant Cell Rep. 21: 517-524.

Hawbaker MS, Fehr WR, Mansur LM, Shoemaker RC (1993). Genetic variation for quantitative traits in soybean lines derived from tissue culture. Theor. Appl. Genet. 87: 49-53.

Heinze B, Schmidt J (1995). Monitoring genetic fidelity vs. somaclonal variation in Norway spruce (*Picea abies*) somatic embryogenesis by RAPD analysis. Euphytica 85: 341-345.

Isabel N, Boivin R, Levseur C, Charest PM, Bousquet J, Tremblay FM (1995). Evidence of somaclonal variation in somatic embryo-derived plantlets of white spruce (*Picea glauca Moench. Voss*). Pages 247-252 in M Terzi, R Cell, A Falavigna, eds. Current issues in plant molecular and cellular biology. Kluwer Academic, Dordrecht.

Kanita A, Kothari SI (2002). High efficiency adventitious shoot bud formation and plant regeneration from leaf explants of *Dianthus chinensis* L. Sci. Hort. 96: 205-212.

Larkin PJ, Scowcroft WR (1981). Somaclonal varaiation – a novel source of variability from cell cultures for plant improvement. Theor. Appl. Genet. 60: 197-214.

Masmoudi R (1999). L'embryogenèse somatique chez le palmier dattier (*Phoenix dactylifera L.*) : induction et maintien des structures embryogènes, caractérisation biochimique.Thèse de Doctorat. Université de Sfax, p. 113.

Michaux-Ferrière N, Grout H, Carron MP (1992). Origin and ontogenesis of somatic embryos in *Hevea brasiliensis* (Euphorbiaceae). Am. J. Bot. 79: 174-180.

Murashige T, Skoog F (1962). A revised meduim for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant 15: 473-497.

Ostry M, Hackett W, Michler C, Serres R, McCown B (1994). Influence of regeneration method and tissue source on the frequency of somatic variation in Populus to infection by *Septoria musiva*. Plant Sci. (Limerck) 97: 209-215.

Rotino GL, Schiavi M, Vicini E, Falavigna A (1991). Variation among androgenetic and embryogenetic lines of eggplant (*Solanum melongena L.*). J. Genet. Breed 45: 141-146.

Sambrook J, Fritsch EF, Maniatis T (1989). Molecular cloning: a laboratory manual. 2nd ed. Cold Spring Harbour Laboratory, Cold Spring Harbor, New York.

Sharma DR, Kumari R, Chowdhury JB (1980). *In vitro* culture of female date palm (*Phoenix dactylifera L.*) tissues. Euphytica 29: 169-174.

Tautorus TE, Fowke LC, Dunstan DI (1991). Somatic embryogenesis in conifers. Can. J. For. Res. 69: 1873-1899.

Taylor PW, Fraser TA, Ko H-L, Henry RJ (1995). RAPD analysis of

sugarcane during tissue culture. Pages 241-246 in Terzi M, Cella R, Falavigna A eds. Curr. Issues Plant Mol. Cell. Biol. Kluwer Academic, Dordrecht.

Touchet B (1991). Micropropagation du palmier à huile (*Elaeis guineensis Jacq.*) en milieu liquide. Thèse de Doctorat. Université de Paris Sud, Orsay, p 153.

Trifi M, Rhouma A, Marrakchi M (2000). Phylogenetic relationships in Tunisian date-palm (*Phoenix dactylifera* L.) germplasm collection using DNA amplification fingerprinting. Agronomie 20: 665-671.

Vasil IK (1995). Cellular molecular genetic improvement of cereals. Pages 5-18 In Terzi M, Cell R, Falavigna A, eds. Curr. Issues Plant Mol. Cell. Biol. Kluwer Academic, Dordrecht.

Williams J, Kuberlik A, Livaak K, Rafalski J, Tingey S (1990). DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acid Res., 18: 653.