IN VITRO CLONING OF DATE PALM *PHOENIX DACTYLIFERA* L., CV. DEGLET BEY BY USING EMBRYOGENIC SUSPENSION AND TEMPORARY IMMERSION BIOREACTOR (TIB)

A. Othmani[1], C. Bayoudh[2], N. Drira[3] & M. Trifi[1]
Faculté des Sciences de Tunis, Laboratoire de Génétique moléculaire, Immunologie et Biotechnologie, El-Manar Tunis, Tunisia[1]
Centre Régional de Recherches Agronomiques Oasiennes, Laboratoire de Culture in vitro, Degache, Tunisia[2]
Faculté des Sciences de Sfax, Laboratoire des Biotechnologies Végétales Appliquées à l'amélioration des cultures, Sfax, Tunisia[3]
Corespondence to: Ahmed Othmani
E-mail: degletbey@yahoo.fr

ABSTRACT

The present work is the first report on in vitro regeneration of an elite date palm cultivar, Deglet Bey (Mnakher) through both somatic embryogenesis and direct shoot formation from young leaf explants cultured on MS agar-solidified medium supplemented with 10 mg.l^{-1} 2,4-dichlorophenoxyacetic acid for 8 months. Factors affecting embryogenic callus and shoot initiation, including antioxidants, adsorbents and 2,4-D concentrations, were investigated. Embryogenic suspensions and culture of shoots in temporary immersion bioreactor (TIB) were developed to improve differentiation of embryogenic callus and proliferation of regenerated shoots, respectively. The yield of cotyledonary somatic embryos produced in half-strength MS liquid media, especially when enriched with 2 mg.l^{-1} 2,4-dichlorophenoxyacetic acid, were shown to be greater than in agar-solidified medium, namely 501 versus 29 per 0.5 g fresh weight of embryogenic callus. The culture of shoot clusters in TIB with an immersion frequency of 5 min every 8 h with MS liquid medium containing 0.04 mg.l^{-1} α-naphthalenacetic acid, 0.2 mg.l^{-1} 6-benzylaminopurine 0.02 mg.l^{-1} kinetin for 6 weeks has clearly improved the yield of regenerated shoots per 15 g of shoot clusters. Indeed, it increased 5.5-fold in comparison with that regenerated on agar-solidified medium. For development into plantlets, cotyledonary somatic embryos and regenerated shoots were cultured on MS agar-solidified medium free of 2,4-D and MS medium comprising 0.1 mg.l^{-1} NAA, respectively.

Keywords: 2,4-D; Date palm cv., Deglet Bey; Embryogenic suspension culture; Organogenesis; Somatic embryogenesis; TIB

Abbreviations: 2,4-D: 2,4-dichlorophenoxyacetic acid; ABA: abscisic acid; BA: 6-benzylaminopurine; KT: kinetin; MS: Murashige and Skoog (1962) medium; NAA: α-naphthalenacetic acid; PGR: plant growth regulator; PVP: polyvinylpyrrolidone; CLMSE: cotyledonary leaf of matured somatic embryo; TIB: Temporary Immersion Bioreactor.

Introduction

The date palm, *Phœnix dactylifera* L., is one of the most economically important perennial plants in arid areas of the Middle-East and the North Africa including Tunisia (4), where it is widely cultivated for food and many other commercial purposes.

Conventionally, this palm is propagated from offshoots, which are limited in number (3-8 during the whole life span of date palm cv. Deglet Bey). Thereafter, in mature date palms, lateral buds are devoted to inflorescence production with a few exceptions.

Seed propagation, on the other hand, does not provide true-to-type offspring due to heterozygosity and seedlings require several years to reach the adult stage. Besides, date palm is slow in flowering and fruiting. Therefore, distinguishing male trees from female ones is not possible before approximately 5 years of field cultivation.

The lack of adequate methods for rapid vegetative propagation of elite genotypes has stimulated intensive efforts to develop large-scale micropropagation of date palm protocols using in vitro tissue culture techniques such as somatic embryogenesis and organogenesis, (2, 8) which have been implemented using agar-solidified media. However, these techniques are difficult to automatise as well as high in production cost, making the systems poorly suitable for mass clonal propagation (36).

The use of large-scale liquid cultures and automation have been well documented, and benefits have been shown both for resolving the manual handling of the various stages of micropropagation, decreasing production cost significantly and for better plant performance by allowing a direct contact of the medium throughout the plant material (41).

Considerable evidence have suggested that 2,4-D is the most commonly used auxin to exert a stimulatory effect on in vitro regeneration of plants (20).

The current investigation was conducted to test the effect of adding 2,4-D to MS agar-solidified medium at various concentrations (1.0, 10, 50 and 100 mg.l^{-1}) on plant regeneration through somatic embryogenesis and shoot organogenesis from young leaf explants of date palm cv. Deglet Bey, a recalcitrant cultivar known by its extreme rarity and its very

high date quality (31). In addition, we describe two efficient methods used for scaling up clonal propagation of this cultivar through the exploitation of both somatic embryogenesis using embryogenic suspension cultures and shoot organogenesis by means of shoot culture in the Temporary Immersion Bioreactor (TIB).

Materials and Methods

Plant materials

Young leaves adjacent to the apex from offshoots were sampled from exemplary adult date palm plants (*Phoenix dactylifera* L.) cv. Deglet Bey growing in open fields in El Mahassen located in southern Tunisia.

Establishment of *in vitro* cultures

Using a hatchet and a serrated knife, offshoot leaves and fiber sheaths were removed acropetally. Young leaves 1-3 cm in length were removed from shoot apex after all mature leaves were removed away.

To prevent explants browning, 60 young leaves were transferred to 1-l Erlenmeyer flask containing 500 ml of an antioxidant solution (150 $mg.l^{-1}$ citric acid and 150 $mg.l^{-1}$ ascorbic acid) for 30 min and then cut into explants (≤ 5 mm long) using a sharp sterilized blade.

Explants were surface sterilized by soaking in 0.01% H_gCl_2 for 1 h, rinsed three times with sterile distilled water and cultured on MS media free of plant growth regulators (PGRs) and supplemented with two adsorbents, including activated charcoal and polyvinylpyrrolidone (PVP) at 0.0, 0.1, 0.2, 0.3, 0.5 and 1.0% (w/v) concentrations for 8 weeks. Shortening the subculture period and excision of browning explants parts during culture were also advocated to prevent this problem.

To study the effect of 2,4-D concentration on the morphogenetic responses, 60 young leaves were treated as for prevention of browning. The obtained explants were disinfected as reported above and cultured on MS induction media containing 0.3 $mg.l^{-1}$ AC and 1.0, 10, 50, and 100 $mg.l^{-1}$ 2,4-D (M_1, M_2, M_3 and M_4, respectively).

All media contained 5% (w/v) sucrose and were gelled with 0.7% (w/v) Difco agar. The pH of media was adjusted to 5.7 prior to autoclaving at 1.4 Kg cm^{-2} for 20 min. Cultures were maintained in the dark at 28±2°C and subcultured every 4 weeks for 8 months under the same culture conditions.

Somatic embryogenesis

After the induction period (8 months), the embryogenic callus obtained on M_2 medium were separated from the original explants and transferred to MS agar-solidified medium supplemented with 0.1 $mg.l^{-1}$ 2,4-D (differentiation medium) in order to stimulate differentiation of embryogenic calli into cotyledonary somatic embryos. The M_2 medium was also tested for the same purpose. 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 month. Experiments were repeated at least three times and at least 25 explants were employed per treatment. Subsequent culture stages were exposed to the same temperature and light regime.

In order to initiate suspension cultures, 0.5 g of friable callus that originated from young leaves cultured during 8 months on M_2 medium was minced, filtered through a 500 µm mesh filter and transferred to 250 ml Erlenmeyer flasks containing 50 ml half-strength MS liquid media (L_1, L_2, L_3, L_4 and L_5) supplemented with 0.3 $mg.l^{-1}$ AC and incorporating 0.0, 0.5, 1, 1.5 and 2 $mg.l^{-1}$ 2,4-D, respectively.

Subculturing was done every 5 days by decanting off the old medium and replacing it with fresh medium of the same composition. Every three days, several samples from suspension cultures were isolated and observed under an inverted microscope for the development of somatic embryogenesis. Morphological characteristics of somatic embryogenesis at different development stages were recorded.

To regenerate plantlets, advanced cotyledonary somatic embryos collected from both MS differentiation medium after 2 months of culture and liquid media after 45 days of culture were transferred separately to agar-solidified MS medium free of 2,4-D (germination medium). Subculturing was done every 4 weeks. The number of plantlets regenerated from somatic embryos was counted after 8 weeks of culture.

In order to study the effect of partial desiccation, cotyledonary somatic embryos derived from embryogenic suspensions after 45 days of culture were transferred to sterile Petri dishes (90 x 20 mm) containing three sterile Whatman filter paper disks. Twenty-five embryos were placed in each Petri dish. The dishes were sealed with parafilm and kept at 27±2°C in the dark for 12, 24, 48, 72 and 96 h desiccation. The relative water content of embryos was calculated according to Malabadi et al. (22). After the desiccation treatments, cotyledonary somatic embryos were transferred to germination medium for light culture.

Shoot organogenesis

The entire expanding explants with resultant shoot organogenesis obtained after an induction period of 8 months on M_2 medium were transferred to MS agar-solidified medium deprived of AC and supplemented with 0.04 $mg.l^{-1}$ NAA, 0.2 $mg.l^{-1}$ BA and 0.02 $mg.l^{-1}$ KT (proliferation medium). M_2 medium was also tested for the same purpose. Cultures were maintained and proliferated by subculturing at intervals of 4 to 5 weeks.

In order to improve multiplication and proliferation of shoot clusters, ten grams of shoot clusters induced from young leaves cultured on M_2 medium during 8 months were transferred into temporary immersion bioreactor (TIB) containers (RITA™, Vitropic-Cirad, France) with 200 ml liquid proliferation medium, at an immersion frequency of 3, 5 or 7 min every 8 h for a 6 week culturing period.

Elongated regenerated shoots derived from both TIB system after 6 weeks of culture and proliferation medium after 3 months of culture were divided into single or few shoots and then transferred separately to MS agar-solidified medium

supplemented with 0.1 mg.l^{-1} NAA (rooting medium) for further elongation and root development during 2 months, and finally transplanted ex vitro into the greenhouse.

Plant acclimatization

Two-month-old healthy regenerated plantlets with well-developed shoots and roots were removed individually from the vessels and the remaining agar was washed away carefully under running tap water. They were then planted in a potting mixture made of peat moss and sand (2:1 ratio) and cultivated in a greenhouse under natural sunlight with 25±2°C temperature and 80-90% relative humidity.

The survival efficiency, defined as the percentage of plants that survived the transfer from in vitro to ex vitro growth conditions, was determined 10 weeks after the plants were placed in the greenhouse.

Statistical analysis

Each treatment was repeated three times. Statistical significance was determined by one-way analysis of variance (ANOVA), followed by Newman and Keuls' test (17, 26) at $P = 0.05$ level.

Results and Discussion

Prevention of browning

In date palm cultivar Deglet Bey, cultivated explants from young leaves turned brown and most of them died especially during the initial stage of explants culture. This result suggests that, the most lethal browning components are produced during this stage.

According to Zaid (37), browning of the date palm tissue and the adjacent medium is assumed to be due to the oxidation of phenolic compounds and formation of quinines which are toxic to the tissues. Browning caused by the oxidation of phenolics has also been reported in cultured apple (34) and *Quercus sp*. (5).

TABLE 1

Comparison of two antioxidant compounds on the survival rate of young leaf explants of date palm cv. Deglet Bey*

Concentration (g.l^{-1})	Survival of explants (%) antioxidant type	
	AC	PVP
0.0	**0 e	**0 a
0.1	5.66 d	0 a
0.2	14 b	0 a
0.3	34 a	0 a
0.5	10.66 c	0 a
1.0	3 e	0.33 a

*All explants treated with an antioxidant solution (150 mg.l^{-1} citric acid + 150 mg.l^{-1} ascorbic acid) and cultured on MS medium free of 2,4-D; data recorded after 8 weeks in culture

**Means followed by the same letter in the same column are not significantly different as indicated by Newman-Keuls' test at $P = 0.05$

A procedure combining pre-treatment of the explants with an antioxidant solution (150 mg.l^{-1} citric acid and 150 mg.l^{-1} ascorbic acid) prior to the surface sterilization treatments and culture on MS medium containing 0.3 mg.l^{-1} AC was found the best method to avoid this phenomenon (**Table 1**). Yet, under these conditions no callus or shoot was produced and most of the explants died after 3 months of culture.

The beneficial effect of citric acid and ascorbic acid on prevention of explants browning is consistent with reports on date palm (38, 39). The effect of AC on minimizing toxic browning of date palm explants has been previously described by Sharma et al. (33). Also, AC is widely used in tissue culture media, where it is believed to function as an adsorbent for toxic metabolic products and residual PGRs (30).

Addition of another adsorbent to nutrient media, such as PVP was ineffective against explants and medium browning (**Table 1**). Similarly, transfer of explants at the onset of browning to a fresh medium *i.e.* at an interval of 10 days and excision of browning explants parts during culture did not prevent explants losses due to browning.

On the basis of this preliminary study, the MS medium supplemented with 0.3 g.l^{-1} AC was selected to study the effect of different concentrations of 2,4-D on the morphogenetic responses obtained from two explants sources, including young leaves and lateral buds.

Somatic embryogenesis

Our experiments revealed that young leaf explants exhibited morphogenetic responses which were found to depend on the 2,4-D concentration.

When the induction medium was supplemented with 1 mg.l^{-1} 2,4-D (M_1), no callus or shoot was produced and most of the explants remained green and died after 8 weeks of culture. Conversely, explants growing on induction medium containing 10 mg.l^{-1} 2,4-D (M_2) gave rise to optimal initiation of embryogenic calli which were friable and showing small (< 2 mm) white nodules (**Fig. 1A**) as well as direct embryogenesis (**Fig. 1B**) and direct shoot formation from the base of the leaves (**Fig. 1C**) within 8 months of culture. This was indicative of the different modes of action of 2,4-D.

Explants growing on medium containing 50 mg.l^{-1} 2,4-D (M_3) could also initiate some callus at the cut ends of explants which were white, hard and non-embryogenic within 4 months. Yet, with culture time prolongation to 7 months, the calli proliferated normally and gave rise to few nodular somatic embryos. However, no signs of callus production or shoot organogenesis were observed in explants developed in the presence of 100 mg.l^{-1} 2,4-D (M_4).

As in the case of date palm cultivar Amsekshi (32), Jihel and Bousthami Noir (42), and Deglet nour (13), it was found that 2,4-D was necessary to induce somatic embryogenesis from leaf explants of cultivar Deglet Bey. But, the concentration of 2,4-D required vary with different genotypes of date palm. This result concurs with previous studies on induction of somatic embryogenesis in a different date palm cultivar (6) and in some other plants such as vetiver (23), sugarcane (12) and Burma

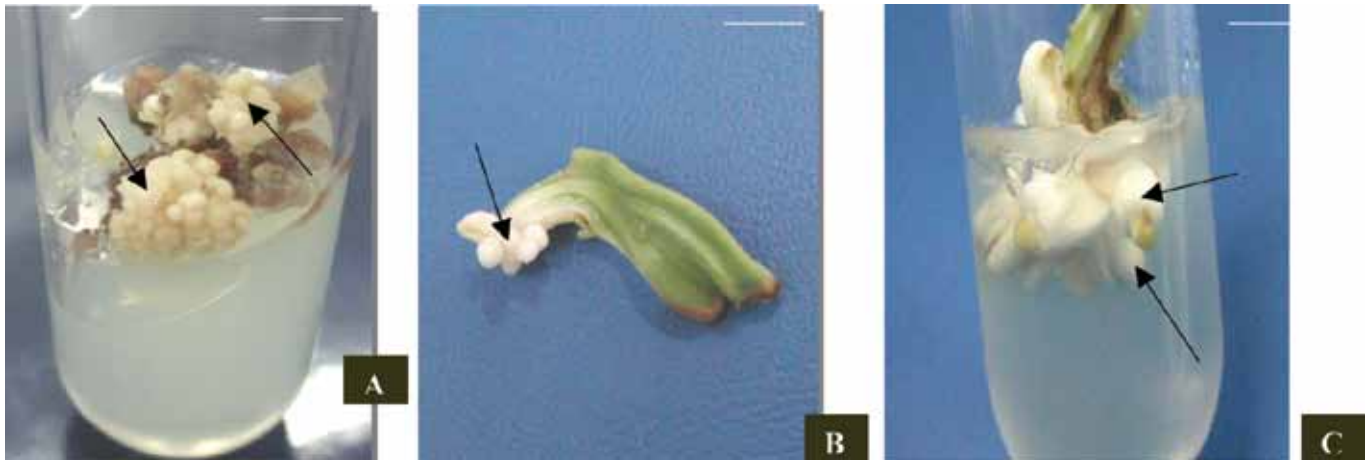

Fig. 1. A-B-C Morphogenesis from young leaf explants of date palm cv. Deglet Bey cultured on MS medium supplemented with 10 mg.l^{-1} 2,4-D after 8 months. **A.** Embryogenic calli (arrows). **B.** Direct embryogenesis (arrow) from the base of a young leaf. **C.** Multiple shoots (arrows) induced from the base of a young leaf. Scale bar: 5 mm (A-B); 8 mm (C)

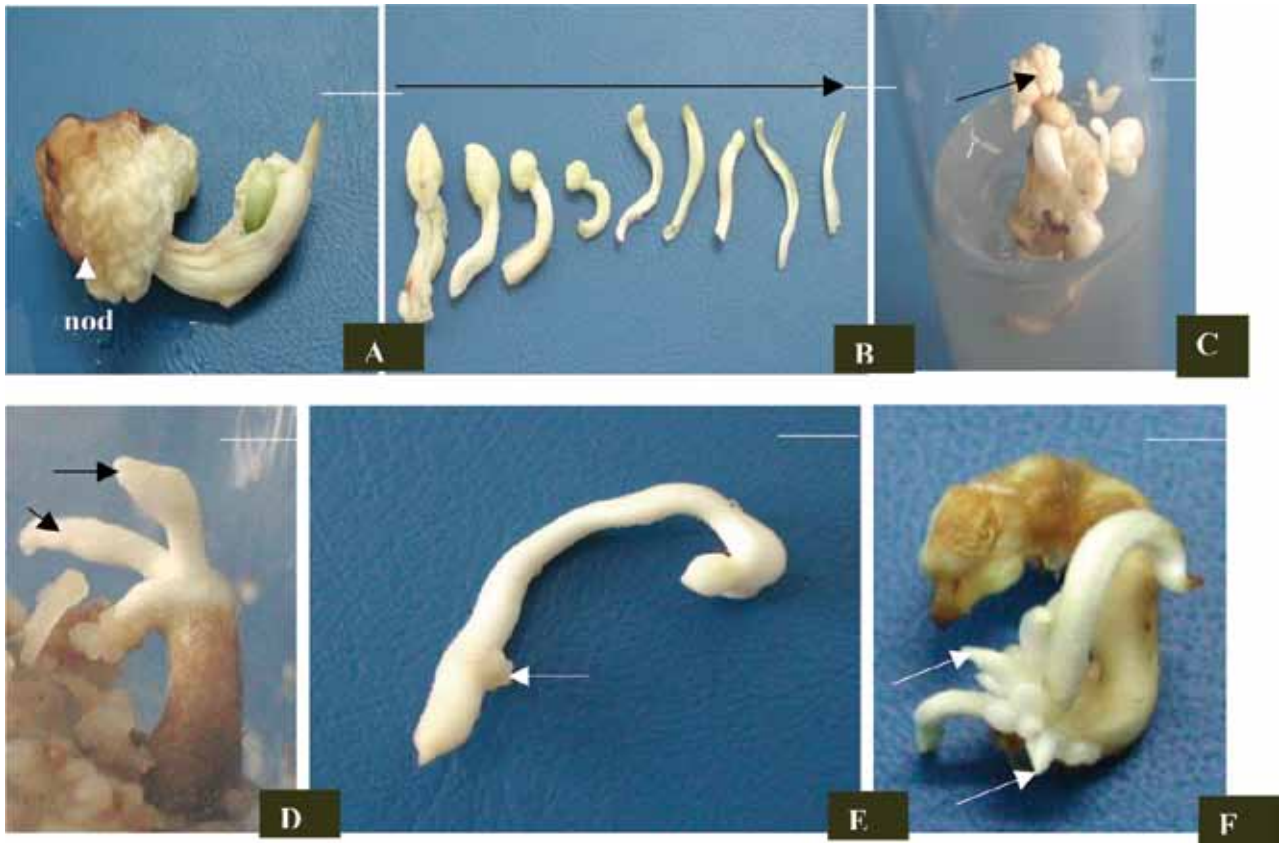

Fig. 2. A-B-C-D-E-F Morphogenetic responses observed on the surface of CLMSEs of date palm cv. Deglet Bey. (**A-B-C-D**) Morphogenetic responses observed on the tip of CLMSEs. **A.** Specific nodule (nod) on MS medium free of 2,4-D for 4 weeks. **B.** Progressive degeneration of nodule (arrow) after 4 subcultures on MS medium free of 2,4-D. **C.** Direct induction of somatic embryos (arrow) from a nodule after 2 subcultures on MS medium with 0,1 mg.l^{-1} 2,4-D. **D.** Differentiation of shoots (arrows) from a nodule after 2 subcultures on MS medium with 0,1 mg.l^{-1} 2,4-D. **E.** Induction of embryogenic callus (arrow) on the median part of CLMSE after 2 subcultures on MS medium with 0,1 mg.l^{-1} 2,4-D. **F.** Induction of shoots (arrows) from the basal party of CLMSE after 2 subcultures on MS medium with 0,1 mg.l^{-1} 2,4-D. Scale bar: 10 mm (A-B); 6 mm (C-D); 5 mm (E-F)

Fig. 3. A-B-C Different stages of somatic embryogenesis in date palm cv. Deglet Bey in suspension culture on half-strength MS liquid medium with 2 mg.l^{-1} 2,4-D. **A.** Globular-stage somatic embryos observed after culturing for 7d. **B.** Elongated embryos observed after 12 d in culture. **C.** Cotyledonous-stage embryos observed within 26 d in culture. Scale bar: 15 mm

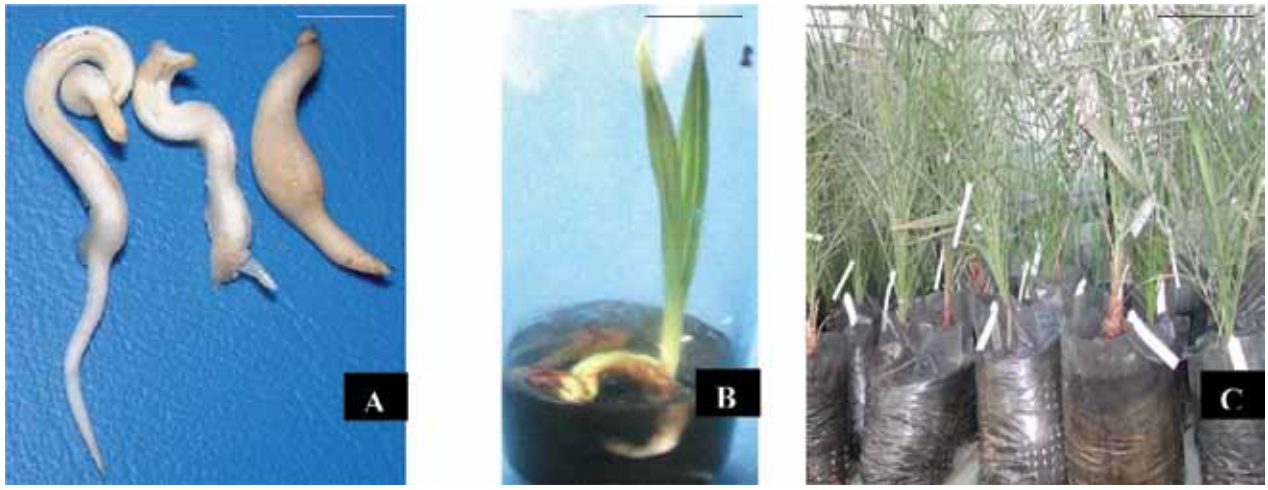

Fig. 4. A-B-C Plants regeneration in date palm cv. Deglet Bey. **A.** Germination of embryos on MS agar-solidified medium deprived of 2,4-D. **B.** plantlet derived from somatic embryos on MS agar-solidified medium deprived of 2,4-D. **C.** Hardened plants 2 years after ex vitro transfer to the green house. Scale bars: 5 mm (A); 10 mm (B); 150 mm (C)

reed (21) in which embryogenesis occurred only in the absence of 2,4-D.

Concerning the differentiation of the embryogenic calli initiated on M_2 medium into cotyledonary somatic embryos, it was recorded that when they were transferred to the same medium for subculture, they grew further and gave rise to yellow-white friable callus. However, no somatic embryos were visible on the callus within 2 months of culture. As the callus was transferred to the differentiation medium, 6% of yellow-white callus proliferated normally and then an average of 29 somatic embryos was observed within 2 months after transfer of 0.5 g FW of embryogenic callus. The positive effect of lowering the 2,4-D concentration on the differentiation of embryogenic calli into cotyledonary somatic embryos was previously observed in date palm cultivars Kenta and Allig (8).

Interestingly, in date palm cultivar Deglet Bey, CLMSEs (cotyledonary leaves of matured somatic embryos) showed numerous morphogenetic responses from all parts of its surface. The tip of every leaf usually developed a specific nodule (**Fig. 2A**). The fate of the latter depended on the 2,4-D concentration; in fact, on a free-PGRs MS medium, nodules' growth decreased until their total degeneration (**Fig. 2B**). However, the addition of 0.1 mg.l^{-1} 2,4-D to MS nutrient medium stimulated indirect or direct embryogenesis (**Fig. 2C**) as well as shoot (**Fig. 2D**) or root organogenesis. Hence, tissues in this region have probably kept the meristematic characteristics of the embryo axis.

On the same medium, the median part of leaves occasionally developed direct or indirect embryogenesis (**Fig. 2E**), but their basal part was the best tissue for the induction of all the types of structures previously described (**Fig. 2F**). Thus, CLMSEs can be used as an abundant source of explants to overcome the rarity and the recalcitrance of Deglet Bey. These results agree with a previous report (15) on the adequacy of such organ for *Manihot glaziovii* Muell. Arg. (*ceara rubber*) micropropagation via secondary embryogenesis.

In our study, we found a gradual decline in the capacity of CLMSEs to form new structures as the embryos matured. However, the excision of CLMSE followed by its transfer into the dark for one month on MS medium containing 0.5

mg.l^{-1} 2,4-D stimulated secondary embryogenesis. Thus, this procedure was found to promote the rejuvenation of cultures.

Following transfer into liquid medium cells from calli exhibited an intense division and gave rise to a highly heterogeneous suspension comprising cells showing differences in shape, size and characteristics.

Microscopic observations of suspension cultures showed that somatic embryos developed from typical globular embryos (**Fig. 3A**) over a period of 7 days, globular-stage embryos further differentiated into elongated embryos within 12 days (**Fig. 3B**). Cotyledonary embryos (**Fig. 3C**) were formed in the next 26 days.

In the liquid culture conditions, all embryo stages were observed in the same culture, this heterogeneity could be explained by the setting of an active and precocious secondary embryogenesis (42). Additionally, our data showed that increasing the 2,4-D concentration in the liquid media from 0.5 to 2 mg.l^{-1} was beneficial to induce somatic embryo proliferation (**Table 2**), a performance better than that previously reported for date palm cv. Deglet Nour (13) and for oil palm (7).

TABLE 2

Effect of medium type and 2,4-D concentration on the number of cotyledonary somatic embryos obtained from 0.5 g of embryogenic callus after 45 d culturing on liquid media L_1, L_2, L_3, L_4 and L_5 and after 2 months culturing on differentiation medium

Medium type		Number of cotyledonary somatic embryos
Liquid medium	*L_1	**0 e
	L_2	341 c
	L_3	397 b
	L_4	407 b
	L_5	501 a
Agar-solidified medium		29 d

*L_1, L_2, L_3, L_4 and L_5: half-strength MS liquid suspension culture media containing 0.4 mg.l^{-1} activated charcoal and (0.0, 0.5, 1.0, 1.5 and 2.0) 2,4-D, respectively

**Means followed by the same letters are not significantly different as indicated by Newman-Keuls' test at $P = 0.05$

In this research, germination of cotyledonary somatic embryos (**Fig. 4A**) obtained on M_2 medium and subsequent conversion into plantlets with fully-developed shoots and roots (**Fig. 4B**) was possible after complete removal of 2,4-D from the differentiation medium. Similar results have been reported by Thiruvengadam et al. (35), with somatic embryos of *Momordica charantia* L. and by Aberlenc-Bertossi et al. (1), with somatic embryos of oil palm. Al-Khayri and Al-Bahrany (2) reported that date palm somatic embryos developed into plantlets on MS medium free of PGRs and containing $AgNO_3$.

It should be noted that culture inducing differentiation of embryogenic calli in agar-solidified or liquid medium strongly affected the germination and conversion rate of cotyledonary somatic embryos into plantlets when transferred to agar-solidified MS medium deprived of 2,4-D. Indeed, 65% of cotyledonary somatic embryos derived from agar-solidified medium germinated and converted into plantlets rather than 22% from suspension cultures.

The low germination rate of cotyledonary somatic embryos derived from suspension cultures is probably due to the inhibitory action of hyperhydricity. It is known that the hyperhydricity phenomenon is major problem for regeneration of diverse species cultured in liquid or in agar-solidified media (29). Kevers et al. (18) reported that hyperhydration is due to a deficient lignification caused by a serious reduction of peroxydases and phenylalanine ammoniaclyase activities.

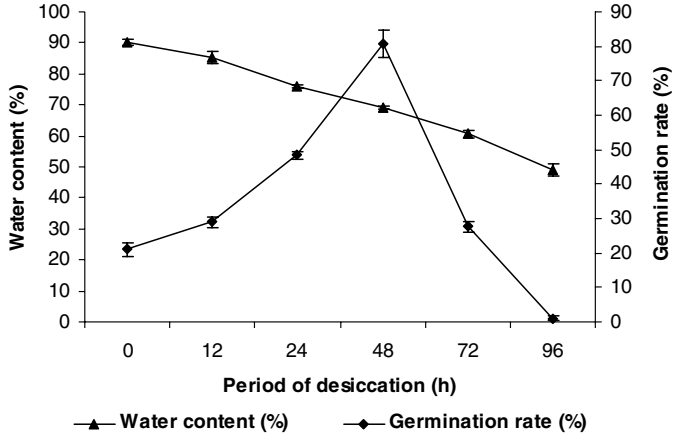

Fig. 5. Effect of desiccation period on the germination rate of cotyledonary somatic embryos derived from L_2 medium when cultured on regeneration medium during 4 weeks. Error bars represent the standard deviation of 3 repetitions

Partial desiccation of mature somatic embryos, corresponding to a decrease in water content from 90.33% to 69% followed by their culture on regeneration medium during 4 weeks, resulted in a significantly higher germination rate (from 21% to 80.66%) (**Fig. 5**). These results confirm those from Fki et al. (13) on the germination of date palm cv. Deglet nour somatic embryos derived from embryogenic suspensions. Monsalud et al. (24) reported that *Mangifera indica* somatic embryos regenerated from suspension cultures germinated precociously when partially dehydrated. According to Jens (14), partial desiccation of somatic embryos induce a substantial decrease in endogenous levels of ABA, a plant growth regulator with a positive effect in the maintenance of embryos in a maturation stage through preventing precocious germination (28)Shoot organogenesis

Of the induction media (M_1, M_2, M_3 and M_4) tested for clonal propagation of date palm cv. Deglet Bey, only the induction medium M_2 was capable of stimulating shoot induction. The subculture of regenerated shoots on the same medium during 3 months resulted in a severe decline in growth and few shoots (5%) were able to elongate. Nevertheless, transferring of shoots to proliferation medium resulted in a relative adventitious proliferation and elongation of axillary buds. Under these conditions, an average of 54 shoots was obtained

from 10 g FW of shoot clusters after 3 months of culture. When such regenerated shoots were subcultured on rooting medium during 2 months, they initiated adventitious roots that attained about 67%.

Application of the TIB for in vitro regeneration through organogenesis or somatic embryogenesis has been described for many crops such as coffee (10), pineapple (9), tea (3), banana (19) and apple (40). However, to our knowledge there is no report describing the use of this technology for the multiplication of date palms. We determined in preliminary experiments (data not shown) that the embryogenic calli of cv. Deglet Bey failed to grow in the TIB and usually turned brown and died. Therefore, we focused our study on the use of shoot cultures in this system in order to generate plantlets.

Fig. 6. Multiple shoot induction (arrows) from shoots obtained on agar-splidified medium that were cultured during 2 weeks in the TIB system containing proliferation medium. The immersion frequency was 5 min every 8 h. Scale Bar: 0.5 mm

After their transfer to the TIB system, shoots elongated faster and gradually produced new shoots as long as they were kept in the regeneration medium (**Fig. 6**) during 6 weeks in comparison with those cultured on agar-solidified medium particularly when immersion was applied for 5 min every 8 h (**Table 3**)The current result confirmed earlier observations made by Peak and Hahn (27), who reported that TIB technology provides the potential for producing large numbers of plants economically and efficiently. This improvement of plant performance in the TIB system is due to a direct contact of the medium with the plant material, a renewing of the culture atmosphere on each immersion and to a reduction of asphyxia, necrosis and tissues hyperhydration phenomena (11)When kept in the TIB container, roots were scarcely produced from adventitious shoots (10%). But, when transferred to rooting medium, elongated shoots exhibited prolific adventitious rooting that reached 96% after 1 month of culture. This result is in contrast with that reported by Kati and Sirpa (16) on strawberry cv. Jonsok for which rooting was optimal when shoots were kept in the TIB containerPlants acclimatization

Transplanting ex vitro and acclimatization of plantlets derived from somatic embryos were established in the greenhouse and 542 surviving plants from 900 (60.2% success rate) acclimatized somaplants were obtained within 3 months (**Fig. 4C**).

On the other hand, transplanting ex vitro and subsequent acclimatization for a period of 2 months of plantlets derived from shoots were sufficient to promote the establishment of plants in the greenhouse where 817 surviving plants were recovered from 850 acclimatized somaplants (96% success rate). For the observed morphological traits and the yield characteristics, no significant differences were seen between parental plants and in vitro derived plants.

It was of interest that plants derived from TIB grew faster and rooted earlier than those derived from agar-solidified medium and suspension culture.

Conclusions

The most important result of this study was the development of an efficient protocol for large-scale micropropagation cultivar Deglet Bey in a shorter period of time. Successful establishment of such a protocol would provide a technique for efficient plant regeneration of other recalcitrant cultivars and thus facilitate

TABLE 3

Effect of medium type on the number, percent hyperhydration and percent necrosis of shoots obtained from 10 g FW of shoot clusters after 6 weeks of culture on proliferation medium

Medium type		Number of shoots	Hyperhydration rate	Necrosis rate
Liquid medium	*TIB_1	**255 b	**3.1 d	**5.03 b
	*TIB_2	284 a	6.0 c	4.96 b
	*TIB_3	220 c	10.16 b	7.00 b
Agar-solidified medium		54 d	18.00 a	29.66 a

*TIB_1 immersion frequency: 3 min/8 h

*TIB_2 immersion frequency: 5 min/8 h

*TIB_3 immersion frequency: 7 min/8 h

**Means followed by the same letter in the same column are not significantly different as indicated by Newman-Keuls' test at $P = 0.05$

much easier vegetative propagation, conservation and genetic engineering of this species.

Acknowledgements

This work was partially supported by grants from the IPGRI «CRPh Degache, Projet FEM-PNUD-IPGRI, RAB 98 G31». We are grateful to Dr. Shri Mohan Jain and Mrs. Ben Maachia Sihem for critically reading the manuscript. Thanks are due to Mr. Hmidi Rchid, Mrs. Ouhibi Besma and Mrs. Sellemi Amel for their technical help.

REFERENCES

1. **Aberlenc-Bertossi F., Noirot M., Duval Y.** (1999) Plant Cell Tissue Organ. Cult., **56**, 53-57.
2. **Al-Khayri J.M., Al-Bahrany A.M.** (2004) Sci. Horticult., **99**, 153-162.
3. **Akula A., Becker D., Bateson M.** (2000) Plant Cell Rep., **18**, 1140-1145.
4. **Awad M.A.** (2007) Acta Horticult., **8**, 129-134.
5. **Bellarosa R.** (1988) Acta Horticult., **227**, 433-435.
6. **Bhaskaran S., Smith R.** (1992) Plant Cell Rep., **12**, 22-25.
7. **De Touchet B., Duval Y., Pannetier C.** (1991) Plant Cell Rep., **10**, 522-532.
8. **Drira N., Benbadis A.** (1985) J. Plant. Physiol., **119**, 227-235.
9. **Escalona M., Lorenzo J.C., González B., Daquinta M., González B., Desjardins Y., Borroto C.G.** (1999) Plant Cell Rep., **18**, 743-748.
10. **Etienne-Barry D., Bertrand B., Vasqez N., Etienne H.** (1999) Plant Cell Rep., **19**, 111-117.
11. **Etienne H., Berthouly M.** (2002) Plant Cell Tissue Organ. Cult., **69**, 215-231.
12. **Fitch M.M.M., Moore P.H.** (1993) Plant Cell Tissue Organ. Cult., **32**, 335-343.
13. **Fki L., Masmoudi R., Drira N., Rival A.** (2003) Plant Cell Rep., **21**, 517-524.
14. **Jens F.** (1997) Plant Sci., **128**, 75-83.
15. **Joseph T., Yeoh H.H., Loh C.S.** (2000) Plant Cell Rep., **19**, 535-538.
16. **Kati J.H., Sirpa O.** (2007) BMC Biotechnology, Article available via: http://www.biomedcentral.com/1472-6750/7/11.
17. **Keuls M.** (1952) Euphytica, **1**, 112-122.
18. **Kevers C., Coumans M., Coumans-Gilles M.F., Gaspar T.H.** (1984) Physiol. Plant. **61**, 69-74.
19. **Kosky R.G., Silva M.F., Pérez L.P., Gilliard T., Martinez F.B., Vega M.R., Milan M.C., Mendoza E.Q.** (2002) Plant Cell Tissue Organ. Cult., **68**, 21-26.
20. **Lakshmanan P., Danesh M., Taji A.** (2002) J. Horticult. Biotech., **77**, 158-163.
21. **Ma G.H., Xu Q.S.** (2002) Plant Cell Tissue Organ. Cult., **70**, 281-288.
22. **Malabadi R., Choudhury H., Tandon P.** (2004) Sci. Horticult., **102**, 449-459.
23. **Marco M., Marisa G., Silvano S.** (1993) Plant Cell Tissue Organ. Cult., **35**, 267-271.
24. **Monsalud M.J., Mathews H., Litz R.E., Gray D.J.** (1995) Plant Cell Tissue Organ. Cult., **42**, 195-206.
25. **Murashige T., Skoog F.** (1962) Physiol. Plant., **15**, 473-497.
26. **Newman D.** (1939) Biometrika, **31**, 20-30.
27. **Peak K.Y., Hahn S.H.** (2001) In Vitro Cell Dev. Biol. Plant., **37**, 149-157.
28. **Pliego-Alfaro F., Monsalud M.J.R., Litz R.E., Gray D.J., Moon P.A.** (1996) Plant Cell Tissue Organ. Cult., **44**, 63-70.
29. **Piatczak E., Wielanek M., Wysokinska A.** (2005) Plant Sci., **168**, 431-437.
30. **Pullman G.S., Gupta P.K.** (1991) Method for reproducing coniferous plants by somatic embryogenesis using absorbent materials in the development stage media. U.S. Patent No 5 034 326.
31. **Rhouma A.** (1994) Le patrimoine génétique, **1**, PNUD/FAO/RAB/88/024.
32. **Sané D., Aberlenc-Bertossi F., Gassama-Dia Y.K., Sagna M., Trouslot M.F., Duval Y., Borgel A.** (2006) Anal. Bot., **98**, 301-308.
33. **Sharma D.R., Kumari R., Chowdhury J.B.** (1980) Euphytica, **29**, 169-174.
34. **Standardi A., Romani F.** (1990) HortScience, **25**, 1435-1436.
35. **Thiruvengadam M., Varisai Mohamed S., Yang C.H., Jayabalan N.** (2006) Sci. Horticult., **109**, 123-129.
36. **Vinocur B., Carmi T., Altman A., Ziv M.** (2000) Plant Cell Rep., **19**, 1146-1154.
37. **Zaid A.** (1987) Acta. Horticult., **212**, 561-566.
38. **Zaid A., Tisserat B.H.** (1983a) Date palm journal, **2**, 163-182.
39. **Zaid A., Tisserat B.H.** (1983b) Bot. Mag. Tokyo, **96**, 67-73.
40. **Zhu L.H., Li X.Y., Welander M.** (2005) Plant Cell Tissue Organ. Cult., **8**, 313-318.
41. **Zobayed S.M.A., Saxena P.K.** (2003) Plant Sci., **165**, 463-470.
42. **Zouine J., El Hadrami I.** (2007) Sci. Horticult., **112**, 221-226.