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Cyclic somatic embryogenesis in date palm (Phoenix dactylifera L.). cv Deglet bey (Mnakher)

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Abstract – For the first time, secondary somatic embryogenesis was investigated as an induced process for scaling up the efficiency of vegetative micropropagation of Tunisian date palm (Phoenix dactylifera L.), cultivar Deglet bey (Mnakher). In the first stage, primary somatic embryos were induced on juvenile leaf explants when cultured on Murashige and Skoog solidified medium (1962) supplemented with 10 mg.l-1 2,4-D and 0.3 mg.l-1 activated charcoal. In the second stage, secondary somatic embryos were occurred on whole parts of primary cotyledonary somatic embryos especially cotyledonary leaves when they were isolated and transferred on MS solidified medium supplemented with 0.1 mg.l-1 2,4-D.Similarly, globular and precotyledonary primary embryos obtained from embryogenic suspension cultures in ½ MS liquid medium supplemented with 2 mg.l-1 2,4-D gave rise to secondary somatic embryos but with the highest rate.
Secondary somatic embryos were converted into plantlets when they were transferred on MS medium free of plant growth regulators and successfully acclimated in the greenhouse.

Keywords: Cotyledonary leaves, Date palm, Plantlets, Somatic embryogenesis

1. Introduction

The date palm, Phoenix dactylifera L., is one of the most economically important perennial plants in North Africa and the Middle East, where it always has been looked upon as the main factor determining social, environmental and economic stability in oases zones. Tunisian date palm germplasm is characterized by high genetic diversity, with more than 250 identified varieties (Rhouma 1994). However, this patrimony is seriously menaced by severe genetic erosion due to various biotic and abiotic stresses. Conventional propagation was made by seeds which do not provide true-to-type offspring due to heterozygosity and seedlings require several years to reach the adult stages. Moreover, propagation via offshoots is relatively slow since the mother plant produces a very limited number of offshoots which does not always satisfy the needs in planting material (Othmani et al. 2009a). The methodology which we adopted to clone date palm consists in inducing regenerations after transit through several phases. The roughest phase on which the system in general depends on is the one relative to the establishment of clusters, corresponding to the initiation of the differentiation of the first embryos or shoots from the primary explants. Indeed, it is about an extremely long phase in view of the slowness of the first morphogenetic expressions (Drira et Benbadis, 1985, Fki et al. 2017).
Besides, it is laborious, since the expression of morphogenetic potentialities of tissues is amply influenced by the development stage of organs, their size, their nature, the period of their sampling and the physiological state of the mother plant (Bouguedoura et al. 2016). In addition, the necessity of modulating, perpetually, the plant growth regulator treatments from the point of view, dose and moment of application according to the state of evolution of the cultures and the mode of regeneration adopted makes this phase painful and uncertain (Masmoudi 1999). Finally, considering the restricted number of axillary buds by offshoot, in particular for some good date quality cultivars of which the sampling requires the sacrifice of mother date palm, not always available in sufficient number, the used system of micropropagation complicates then more.
In view of all these considerations, it turns out imperative, to master such system via the extension of the proliferation capacity of the cultures during numerous successive generations of multiplication. It is

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also a question of clarifying the involved processes required in plantlets regeneration by a regular and precise follow-up of the transformations that they underwent, with the aim of making of the in vitro culture a method of choice in the service of micropropagation of date palm. To overcome these drawbacks, in vitro tissue culture techniques such as somatic embryogenesis and organogenesis have been developed to provide an alternative strategy aimed at conservation of date palm genetic resources. Somatic embryogenesis is reported to be a quick and efficient method for large scale propagation of date palm and could also be highly useful for breeding programs and especially for secondary somatic embryogenesis (El Hadrami et al. 1998; Al-Khayri 2011). Secondary somatic embryogenesis or repetitive orcyclic somatic embryogenesis consists of the production of new somatic embryos using primary somatic embryos as initial explants (Raemakers et al. 1995). This process has been described in nearly one hundred species (Kamle et al. 2011). According to Karami et al. (2008), secondary somatic embryogenesis has certain advantages when compared to primary somatic embryogenesis such as a high multiplication rate, independence of an explants source and repeatability. Many research papers have been published on the initial establishment of direct and indirect primary somatic embryogenesis in date palm but the process of secondary somatic embryogenesis has not been investigated (Meziani et al. 2015). The development of such protocol can be successfully employed for the improvement of date palm by providing a rapid and efficient vegetative miropropagation method for ex-situ conservation of elite genotypes via cryopreservation and development of genetic transformation systems. This paper portrays on the efforts made towards inducing secondary somatic embryogenesis in date palm. cv Deglet bey (Mnakher), an extreme rare variety reputed by its high date quality (Othmani et al. 2009b) for use in conservation programs.

2. Materials et Methodes

2.1. Plant material and explant preparation

Juvenile leaves adjacent to the apex from offshoots were sourced from outstanding adult date palm plants (Phoenix dactylifera L.), cv Deglet bey growing in open fields in El Mahassen, southern Tunisia. Juvenile leaves were surface sterilized in 0.01% mercuric chloride for 1h, and then rinsed three times with sterile distilled water.

2.2. Initiation of primary somatic embryos

Juvenile leaves were cut into 1-1,5 cm long explants using a sharp sterilized blade. The explants were inoculated on MS basal medium incorporating 10 mg.l-1 2,4-D, 30 g.l-1 sucrose and 0.3 g.l-1 activated charcoal (M1) and were solidified with 0.7% agar. The pH of all media was adjusted to 5.7 prior to autoclaving at 120°C and 1.4 Kg cm-2 for 20 min. Cultures were kept in darkness at 28 ± 2°C and subcultured every 4 weeks for 6-7 months under the same culture conditions. After this induction period, the embryogenic callus were separated from the original explants and transferred onto MS solidified medium supplemented with 0.1 mg.l-1 2,4-D in order to stimulate the development of embryogenic embryos. Cultures, subcultured monthly, were placed in a 28 ± 2°C culture room with a 16/18 h photoperiod with 80 µmol m-2 s-1 fluorescent light.

Table 1. Media composition used for induction of primary and secondary somatic embryogenesis

Medium composition (mgl^{-1})	**M_1**	**M_2**	**M_3**	**M_4**
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_2PO_4	120	120	120	120
Adenine	30	30	30	30
Difco agar	7 000	7 000	7 000	7 000
2,4-D	10	0,1	0	0,5
Activated charcoal	300	0	0	200

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2.3. Initiation of secondary somatic embryos

To induce secondary somatic embryogenesis, primary cotyledonary somatic embryos regenerated on agar solidified medium were isolated and transferred ontoMS culture medium comprising 0,1 mg l-1 2,4-D (M2). Subsequent culture stages were exposed to the same temperature and light regime.

2.4. Induction of secondary somatic embryogenesis from embryogenic suspension cultures

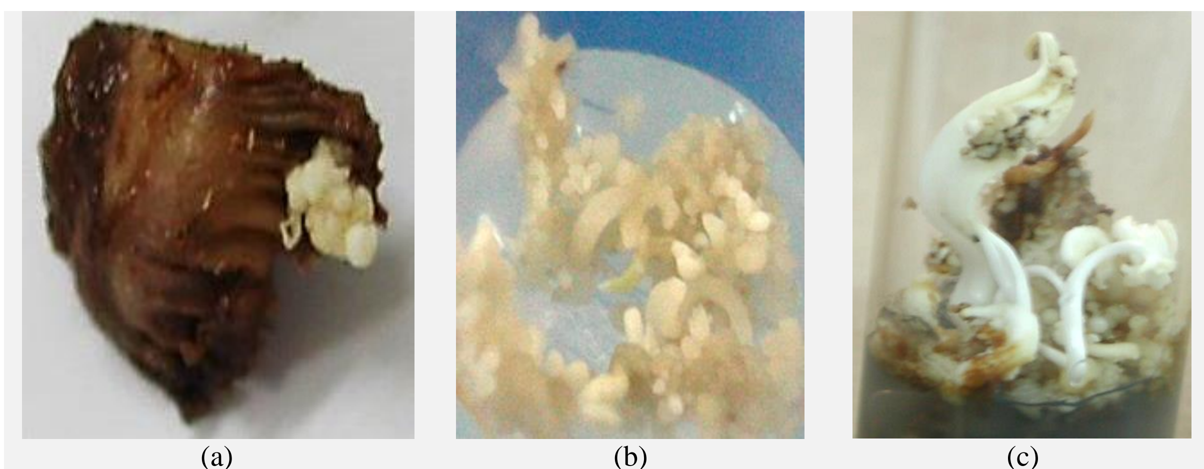
With the aim of initiating embryogenic suspension culture, 0.5 g of friable callus was minced, filtered through a 500 µm mesh filter and transferred to 250 ml Erlenmeyer flasks containing 50 ml half- strength MS liquid medium supplemented with 0,4 mg l-1 activated charcoal and incorporating 2mg l-1 2,4-D (Othmani et al. 2009b). Subculturing was done every 5 days by decanting off the old medium and replacing it with fresh medium of the same composition. Every 3 days, several samples from suspension cultures were isolated and observed under an inverted microscope for the development of both primary and secondary somatic embryos.

2.5. Conversion of somatic embryos into plantlets

To regenerate plantlets, newly formed secondary somatic embryos collected from both agar solidified medium and embryogenic suspension cultures were transferred separately to agar-solidified MS medium comprising 0.1 mg.l-1 NAA. Subculturing was done every 4 weeks.

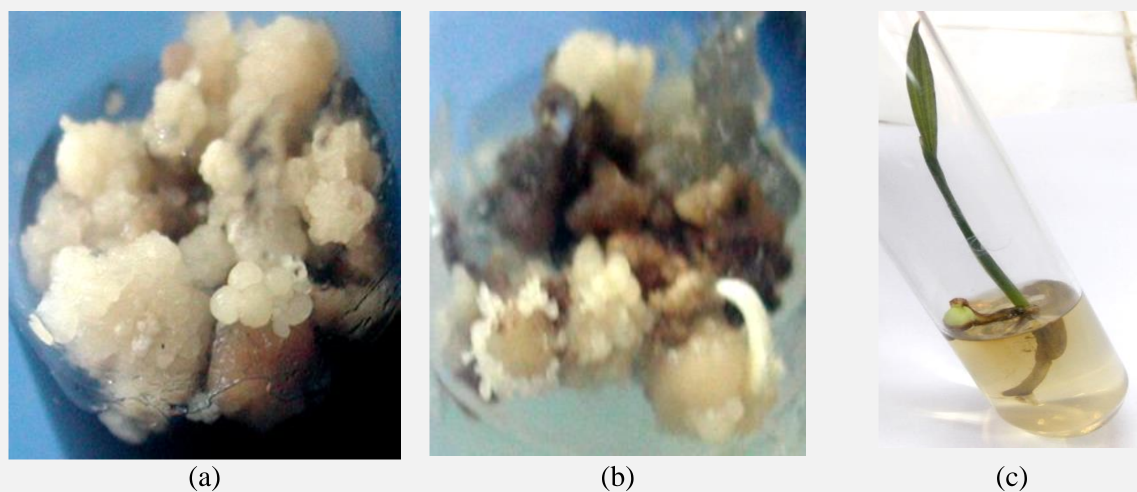
3. Results and Discussion

After 8 months of culture on MS medium containing 10 mg.l-1 2,4-D(M1), juvenile leaf explants gave rise to primary somatic embryos whether after transit by undifferentiated cell culture phase; indirect somatic embryogenesis (Fig 1a, b and c) or in a direct way (Fig 2a, b and c).



(a) (b) (c)

Figure 1. Indirect somatic embryogenesis. a: nodular callus after 8 months of culture on M_1 medium. b: somatic embryos in the process of maturation. c: matured somatic embryos in process of conversion into plantlets.



(a) (b) (c)

Figure 2. Direct somatic embryogenesis. a: embryos on nodular phase after 8 months of culture on M_1 medium. b: somatic embryo in process of maturation. c: vitroplant derived from the conversion of a somatic embryo.

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When the primary somatic embryos were transferred onto MS culture medium comprising 0,1 mg.l-1 2,4-D (M2), they gave rise essentially to de novo somatic embryos (72%). It's a question of secondary somatic embryogenesis process. However, direct and indirect formation of shoots, have occasionally (5%), observed.
Besides, we noted that secondary somatic embryos and shoots in formation appeared essentially from cotyledonary leaf tissues of primary somatic embryos. The latters, showed at the same time multiple and diverse morphogenetic capacities on different levels of their surface.
Interestingly, the tip of every cotyledonary leaf of primary somatic embryos of the studied cultivar develops permanently specific nodules with different forms and sizes (Fig 3).

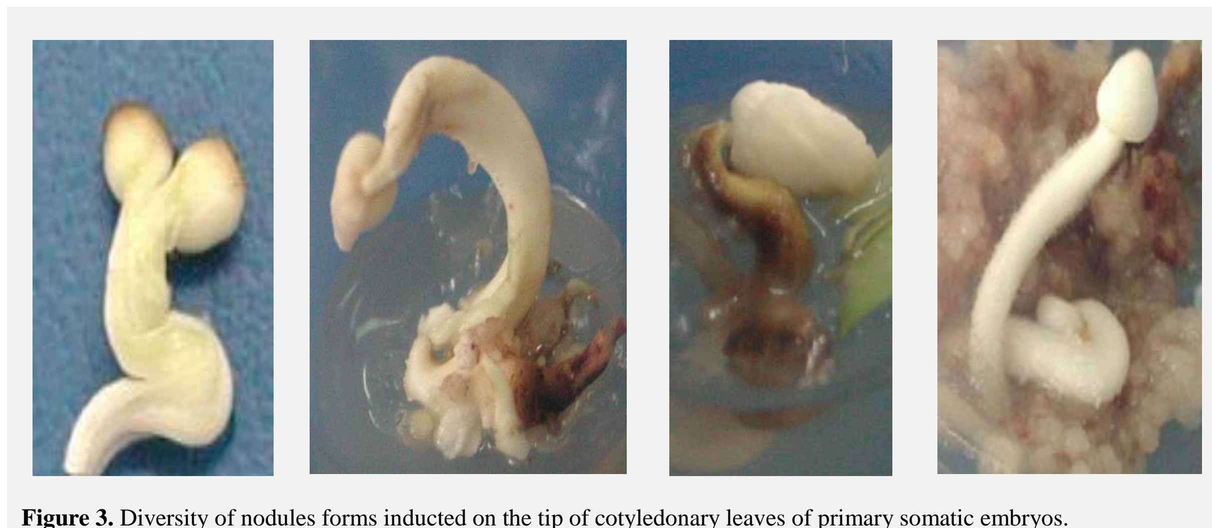
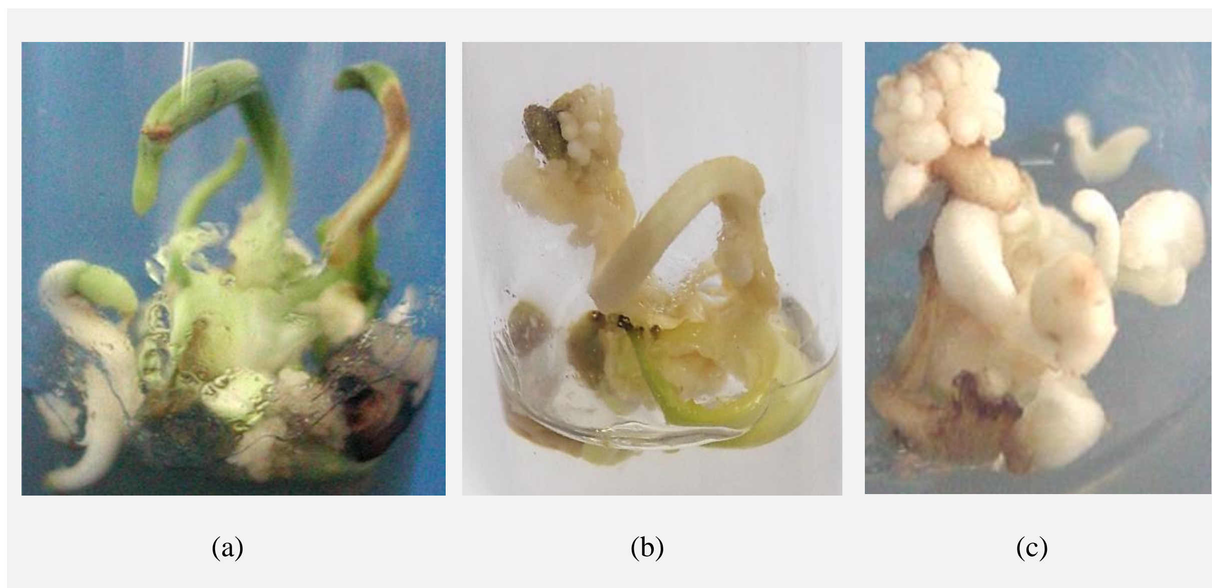
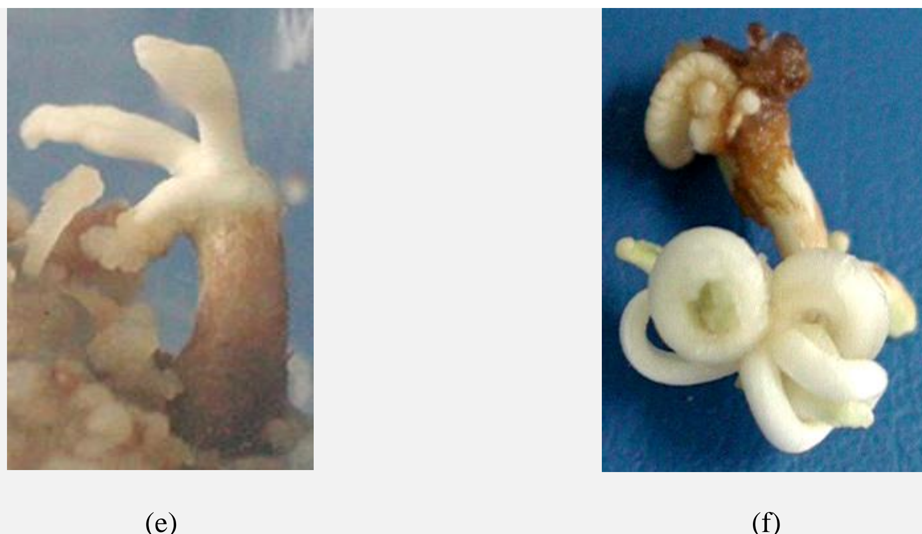


Figure 3. Diversity of nodules forms inducted on the tip of cotyledonary leaves of primary somatic embryos.

The fat of these structures is strictly depended on the 2,4-D concentration. Indeed, on a medium devoid of plant growth regulators (M3), nodules size decreases gradually until their total degeneration (Fig 4a). Nevertheless, when they were maintained on the same medium (M2), nodules gave rise to embryogenic calli (Fig 4b) as well as direct embryogenesis (Fig 4c)and direct shoot organogenesis (Fig 4d) or root organogenesis (Fig 4e).Thus, this result suggests that the tissues in this region have probably reserved the meristematic characteristics of the embryo axis.



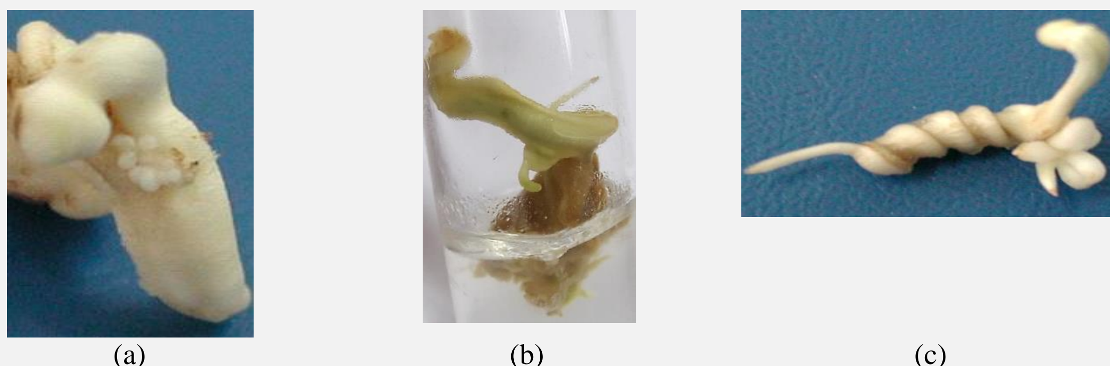
(a) (b) (c)



(e) (f)

Figure 4. Induction of morphogenetic responses from nodules of primary somatic embryos according to the 2,4-D concentration. **a**: degeneration on M_3 medium. **b**: induction of embryogenic callus on M_2 medium. **c**: Direct induction of somatic embryos on M_2 medium. **d**: direct formation of 3 shoots on M_2 medium. **e**: induction of root organogenesis M_2 medium.

As regards for the median part of these leaves, it can develop, sometimes (15%) certain nodular calli (Fig 5 a) when primary somatic embryos were cultured on M2 medium. But, it is especially endowed with a high potentiality (90%) to give rise direct secondary somatic embryos (Fig5b), particularly during the first generation of multiplication on M2 medium. Occasionally, this part of these leaves can develop adventitious shoots (Fig5c).



(a) (b) (c)

Figure 5. Morphogenetic responses recorded on the median part of cotyledonary leaves of primary somatic embryos when cultured on M_2 medium. **a**: Induction of a nodular callus. **b**: Direct induction of somatic embryogenesis. **c**: Direct induction of shoots.

But, their basal part was the best region for the stimulation of all types of structures previously described (Fig 6 a,b).
For this reason, cotyledonary leaves of somatic embryos can be used as an abundant source of explants to overcome the rarity and the recalcitrance of the cultivar Deglet bey.

(a) (b)

Figure 6. Morphogenetic responses recorded on the basal part of cotyledonary leaves of primary somatic embryos when cultured on M_2 medium. **a**: Concomitant induction of callus and shoots. **b**: Direct induction of somatic embryogenesis.

It is advisable to underline that these secondary emissions often appear during the first generations, finish by disappear after 2-3successive multiplication cycles. However, we found that the excision of cotyledonary leaves of primary somatic embryos followed by their transfer into the dark for one month on MS medium supplemented with 0.5 mg.l-1 (M4) stimulated especially secondary somatic embryogenesis. Consequently, such technique permits to promote the rejuvenation of culture during many years.

Rarely, the tip of certain roots of somatic embryo clusters (3%) develop embryogenic callus (Fig7a) or small nodules (Fig7b) which correspond to somatic embryos at the spherical stage when it transferred on M4 medium; it is a question of direct somatic embryogenesis. The spherical stage embryos differentiated to mature somatic embryos after 2 months of transfer onto MS medium deprived of plant growth regulators.

(a) (b)

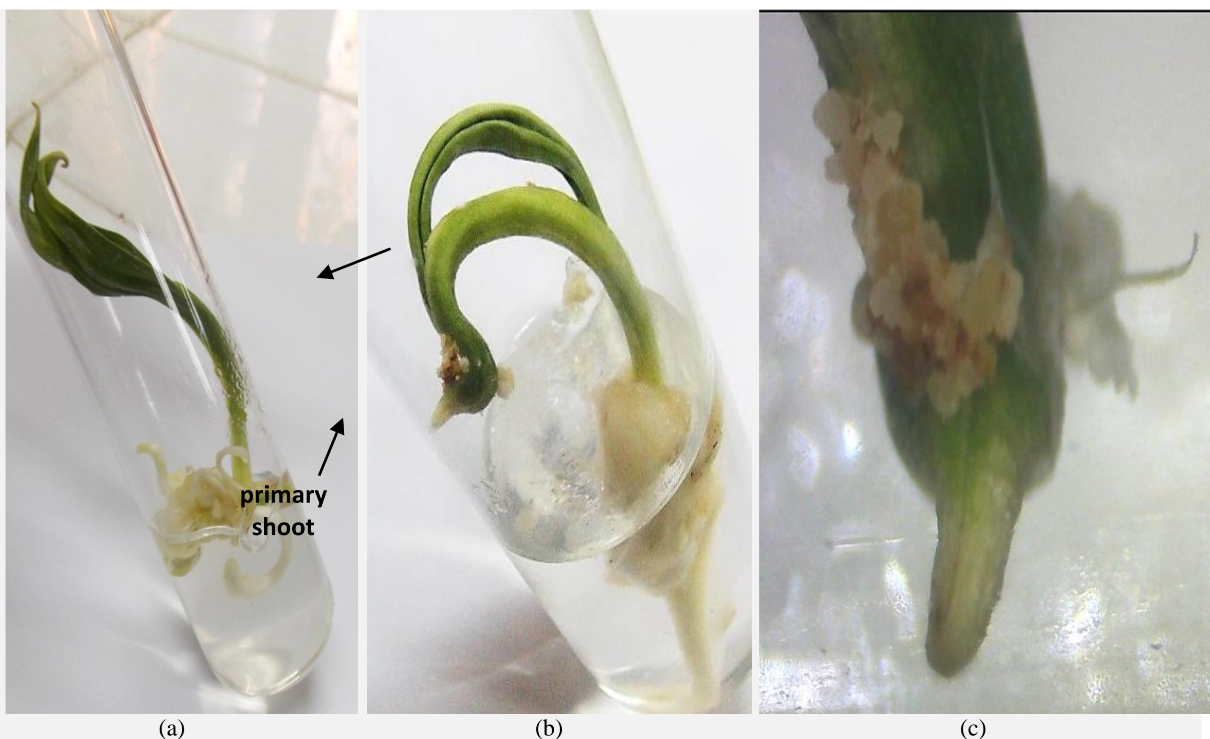
Figure 7. Morphogenetic responses recorded on the tip of certain roots of embryogenic clusters when cultured on M_4 medium. **a**: Induction of an embryogenic callus. **b**: Induction of a nodular callus.

In Parallel to secondary somatic embryogenesis, a secondary shoot organogenesis was also recorded (Fig8a), especially when primary shoots where transferred on M3 medium. The subculture of secondary shoots on MS medium supplemented with ANA and BA (1-1 mg.l-1), insure both multiplication (Fig8b) and development of shoots susceptible of conversion into plantlets after approximately 2 subcultures into M2medium (Fig8c).

(a) (b) (c)

Figure 8. Secondary shoot organogenesis and plantlets formation. **a**: Secondary shoot organogenesis recorded on primary shoots when cultured on MS medium supplemented with ANA and BA (1-1 mg.l^{-1}). **b**: Multiplication of secondary shoots. **c**: vitroplants obtained after rooting of shoots.

The remarkable capacity of proliferation already mentioned in the primary somatic embryos of the cultivar Deglet bey can be retained in certain plantlets that derived from their conversion. Indeed, some of these plantlets are capable to give rise to shoot clusters (Fig 9a) that emerge directly from their shoot apex or embryogenic callus near to the tip of their leaves (Fig 9b and c).



(a) (b) (c)

Figure 9. Morphogenetic responses recorded on plantlets. **a**: Induction of shoots on the apex shoot. **b**: Induction of callus and roots near to the tip of leaves. c: nodular calls and a root observed after enlargement of the tip zone of a plantlet a leaf X 100

The follow-up of the morphological transformations undergone by the embryogenic suspension cultures, allows detecting some aggregates (%) of little nodules corresponding to an undifferentiated secondary somatic embryos that initiated on the surface of the pro-embryos (Fig 10). These secondary somatic embryos get loose easily under the influence of the excitement and they can follow two ways; some of them (30%) develop in structured somatic embryos, others contribute to the establishment of new cycles of multiplication. Such phenomenon is responsible for a significant improvement of the biomass of the culture.

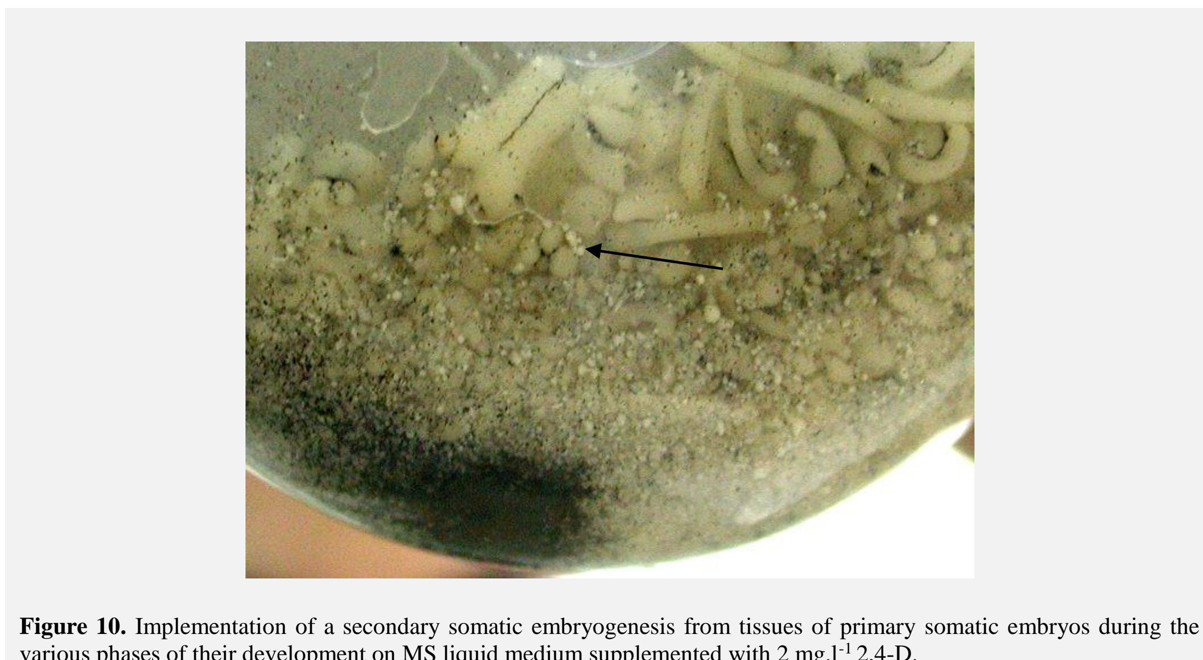


Figure 10. Implementation of a secondary somatic embryogenesis from tissues of primary somatic embryos during the various phases of their development on MS liquid medium supplemented with 2 mg.l^{-1} 2,4-D.

By opposition to the explants taken from offshoots, those derived from in vitro tissue culture, in particular from somatic embryos react rather quickly and seem hypersensitive to the effect of plant growth regulators. Indeed, the low 2,4-D concentrations, which seem generally unfavorable to the induction of the callogenesis in the case of explants derived from offshoots, proved to be, on the other hand, effective in the case of explants taken from in vitro culture. For example, once mature somatic embryos of the cultivar Deglet bey was cultured on MS medium containing 0,1 mg.l-1 2,4-D (M2), they gave rise to diverse types of morphogenetic responses, including secondary somatic embryos.
The secondary somatic embryogenesis is frequent in many species during the phases of maturation and conversion of primary somatic embryos into plantlets, particularly when it is about culture on agar solidified medium (Ziv et Gadasi 1986; Abousalim et Hafdi 1995). According to Wan and Vasil (1982), three to four generations of somatic embryos can be obtained from the primary embryos.
Our experiences proved that diverse modes of regeneration, characterized each one by the nature and the origin of the morphogenetic responses, are highly influenced by the 2,4-D concentration of the employed culture medium during the multiplication phase. According to Masmoudi (1999), the nature and the origin of these responses were also influenced by the composition of the culture media used during the phase of initiation of morphogenetic responses.
As reported by Zouine et El Hadrami (2007), the stimulating effect of the 2,4-D on the secondary somatic embryogenesis could be partially explained by the induction of cellular division at both the apex of the primary somatic embryos and the apical plantlets shoot.
In this context, Nhut et al. (2006) report that the addition of thidiazuron (TDZ) to the culture medium stimulated secondary somatic embryogenesis in Lilium longiflorum.
As for the precise origin of the secondary somatic embryos of the date palm, Zaid (1989) noted that they arise from the reactivation of the epidermic cells of primary somatic embryos. The follow-up of the polyembryonic cultures allowed us to identify diverse modalities of expression of secondary somatic embryogenesis. The latter is frequently induced from all tissues of cotyledonary leaves of primary somatic embryos from the base towards the terminal extremity without distinction of place with however zones and privileged forms of appearance were recorded. It is the case of the tip of cotyledonary leaves

of primary somatic embryos of the cultivar Deglet bey that gave rise a specific nodule, from which diverse types of morphogenetic responses can appear. These results are agree with a previous report of Joseph et al (2000) on the adequacy of such organ for Manihot glaziovii Muell. Arg. (Ceara rubber) micropropagation via secondary somatic embryogenesis.

Certainly, if the regenerated somatic embryos are genetically stable, their cotyledonary leaves can serve as source of sampling explants, in the same way as the offshoots, to overcome the rarity and the recalcitrance of some cultivars of date palm. This was moreover already reported by Masmoudi (1999) in date palm, cultivar Deglet nour, Tivarekar and Eapen (2001) in Vignamungo, Ning and al. (2007) in prunus mume and Seo et al. (2007) in Sesamum indicum L .Besides, Trigiano (1997) mentioned that the secondary somatic embryogenesis inhibited the normal process of primary somatic embryos germination.

The excision of primary somatic embryo cotyledonary leaves, when it doesn't cause an excessive emission of polyphenolic compounds, improved clearly the reactivity of their tissues. This disturbance of the morphogenetic correlations is a strongly inductive factor of a great importance on plant cell differentiation. Also, it favors the putting in direct contact of the competent cells to the somatic embryogenesis with the culture medium.

In parallel, we noticed a callogenesis on the tip of some roots of certain somatic embryos of the cultivar Deglet bey. This unexpected phenomenon was not previously detected in the date palm, but it was observed in other species, such as Arabidopsis thaliana (Sugiyama et Imamura 2006) and Saint John wort (Hypericum perforatumL.) (Zobayed et Saxena 2003).Concerning the organogenic cultures, it multiplied essentially by a secondary formation of new shoots; phenomenon previously described by Drira (1985) and Masmoudi (1999).

As for embryogenic suspension cultures, the fact of having an active secondary somatic embryogenesis, constitutes a decisive advantage in the field of micropropagation of date palm in the industrial scale. Indeed, the number of somatic embryos obtained in agitated liquid medium is upper by far to the one obtained on agar solidified medium from the same mass of embryogenic callus. This benefit effect of liquid culture on the induction of secondary somatic embryogenesis was already reported on date palm by Masmoudi (1999), Zouine et El Hadrami (2007) and Abohatem et al (2017).

4. Conclusion

In conclusion, we demonstrated that secondary somatic embryogenesis is feasible in date palm and it could be used for large scale micropropagation of potential genetic transformed somatic embryos in a shorter time.

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